VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ

BRNO UNIVERSITY OF TECHNOLOGY

FAKULTA CHEMICKÁ
ÚSTAV FYZIKÁLNÍ A SPOTŘEBNÍ CHEMIE

FACULTY OF CHEMISTRY
INSTITUTE OF PHYSICAL AND APPLIED CHEMISTRY

COLLOID PROPERTIES OF HYALURONANE SOLS

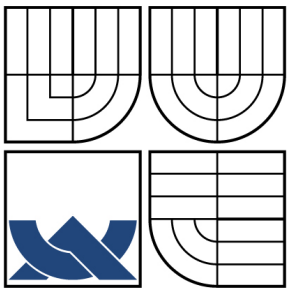
KOLOIDNÍ VLASTNOSTI HYALURONOVÝCH SOLŮ

DIPLOMOVÁ PRÁCE
DIPLOMA THESIS

AUTOR PRÁCE
AUTHOR

JITKA KROUSKÁ

BRNO 2008



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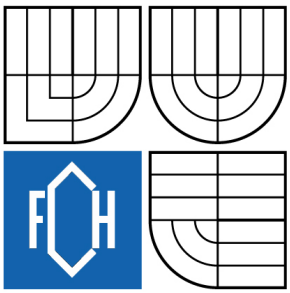

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BRNO 2008

Vysoké učení technické v Brně
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Zadání diplomové práce

Číslo diplomové práce	**FCH-DIP0128/2007**	Akademický rok: **2007/2008**
Ústav	Ústav fyzikální a spotřební chemie	
Student(ka)	**Krouská Jitka**	
Studijní program	Spotřební chemie (M2806)	
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Vedoucí diplomové práce	**doc. Ing. Miloslav Pekař, CSc.**	
Konzultanti diplomové práce		

Název diplomové práce:

Koloidní vlastnosti hyaluronových solů

Zadání diplomové práce:

1. Rešerše na téma povrchové a solubilizační vlastnosti hyaluronových koloidních roztoků.
2. Návrh experimentů studujících uvedené vlastnosti a jejich ovlivnění složením roztoku, popř. teplotou.
3. Provedení experimentů.
4. Vyhodnocení výsledků zejména z hlediska využití hyaluronanů jako nosičů hydrofobních látek.

Termín odevzdání diplomové práce: 16.5.2008

Diplomová práce se odevzdává ve třech exemplářích na sekretariát ústavu a v elektronické formě vedoucímu diplomové práce. Toto zadání je přílohou diplomové práce.

Jitka Krouská	doc. Ing. Miloslav Pekař, CSc.	
student(ka)	Vedoucí práce	Ředitel ústavu

V Brně, dne 1.9.2007

doc. Ing. Jaromír Havlica, CSc.
Děkan fakulty

ABSTRACT

The objective of this thesis was to describe aggregation properties of hyaluronic acid (HA) and its hydrophobically modified derivatives. For this purpose, two HA derivatives in aqueous solutions differing in degree of substitution and anorganic salt environment were prepared. The concentration series of pure HA and its derivatives were studied by two methods, tensiometry and spectrophotometry. The surface properties were measured with Du Noüy ring. The solubilization behaviour in water and sodium chloride media was also investigated with the probes, the Coomassie Brilliant Blue and Sudan III dyes. The result is that the amphiphilic HA derivatives may indeed establish hydrophobic associations in bulk solution while adsorbing at the air–water interface. This property is important when talked about drug delivery systems, because the hydrophobic domains in the aggregates of HA derivatives may be used as a delivery system for hydrophobic drugs. Critical aggregation concentrations of HA derivatives were obtained from tensiometric and spectrophotometric measurement and these concentrations correspond directly to the concentration in which the hydrophobic domains occur.

ABSTRAKT

Předmětem této práce bylo popsat agregační vlastnosti kyseliny hyaluronové (HA) a jejích hydrofobně modifikovaných derivátů. Za tímto účelem byly připraveny vodné roztoky kyseliny a dvou derivátů, lišící se stupněm substituce a druhem anorganické soli jako prostředím. Koncentrační řady čisté kyseliny hyaluronové a jejích derivátů byly studovány dvěma metodami, tensiometricky a spektrometricky. Povrchové vlastnosti roztoků byly měřeny s použitím kroužku Du Noüy. Zkoumáno bylo také solubilizační chování ve vodě a v chloridu sodném pomocí dvou sond: Coomassie brilantní modři a sudanu III. Amfifilní deriváty hyaluronanu mohou skutečně tvořit hydrofobní agregáty v roztoku a adsorbovat se na rozhraní vzduch–voda. Tato vlastnost je důležitá v souvislosti s transportními systémy léčiv, protože právě hydrofobní domény agregátů hyaluronanu mohou být použity jako nosiče hydrofobních léčiv. Byly zjištěny kritické agregační koncentrace roztoků derivátů hyaluronanu (tensiometricky a spektrometricky), které odpovídají právě těm koncentracím, při kterých vznikají hydrofobní domény.

KEYWORDS

hyaluronic acid, hyaluronan, tensiometry, Du Noüy ring, solubilization

KLÍČOVÁ SLOVA

kyselina hyaluronová, hyaluronan, tensiometrie, kroužek Du Noüy, solubilizace

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DECLARATION

I declare that this thesis has been compiled by myself and on my own and I cited all my information sources completely and correctly. The diploma thesis is in terms of its contents a property of the BUT Faculty of Chemistry and its usage for commercial purposes is subject to a prior consent of the supervisor and the dean.

PROHLÁŠENÍ

Prohlašuji, že jsem diplomovou práci vypracovala samostatně, a že všechny použité literární zdroje jsem správně a úplně citovala. Diplomová práce je z hlediska obsahu majetkem Fakulty chemické VUT v Brně a může být využita ke komerčním účelům jen se souhlasem vedoucího diplomové práce a děkana FCH VUT.

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signature of diploma thesis author
podpis diplomanta

CONTENT

1 INTRODUCTION

The beginning of 21st century yields a large development of new branches in biochemistry and medicine. Above all, plastic surgery, tissue engineering and nanotechnologies have become very popular.

The effort of experts is to help the patients who fight with diseases such as cancer, diabetes, etc., therefore, doctors need good drug delivery systems in all these problems. In such treatment, it is necessary to deliver the drugs directly to the infliction cells. For patients with diabetic wounds suitable methods for their healing have been finding. Last, but not least, we talk about tissue implants which substitute the natural ones during any operation, e.g. blood vessels, joints, cartilages, etc.

After the discovery of hyaluronic acid (HA), i.e. a polysaccharide with a wide usage in the branches mentioned above and in many more, patients with cancer, diabetes, joint diseases etc. should get better care. Scientists work on suitable hydrofobically modified hyaluronic acid (derivatives of HA) that is believed to become a new possible drug delivery system for drugs especially against cancer. There are new methods in tissue engineering dealing with "scaffolds" which allow growing e.g. synthetic cartilages and vessels.

This diploma thesis deals with surface tension measurements of HA solutions in various solvents. The aim is to clarify the surface properties of this substance in aqueous environment. The work is focused also on already mentioned derivatives of HA. The second part of the work deals with solibilization properties of dyes in derivatives media.

Both results are given in form of critical aggregation concentration which describes the characteristic features of any surface active substance in the best way.

2 STATE OF THE ART

2.1 Hyaluronic Acid

Hyaluronic acid (HA) was discovered by Karl Meyer and John Palmer in 1934 in the vitreous humor of cattle eyes. HA is a linear, unbranched polysaccharide with a high molecular weight [1]. It ussualy occurs with its sodium salt, sodium hyaluronate, to form hyaluronan. This polysaccharide belongs to a group of substances known as glucosaminoglycans [2].

It is a negatively charged [3] natural polysaccharide formed from disaccharide units composed of alternating (1→4)-β linked D-glucuronic acid and (1→3)-β linked N-acetyl-D-glucosamine residues (Fig. 1). It is negatively charged due to the presence of carboxylic group on the glucuronic unit. The molecular weight (MW) reaches usually 10^5–10^7 Daltons (Da) [2]. For example, HA with MW of 1.63×10^6 Da obtain approximately 4075 repeating units [4]. A pure HA has a very hydrophilic character. Hygroscopicity of dry HA is one of the complicating factors when a solution with precisely defined concentration of HA is required [2].

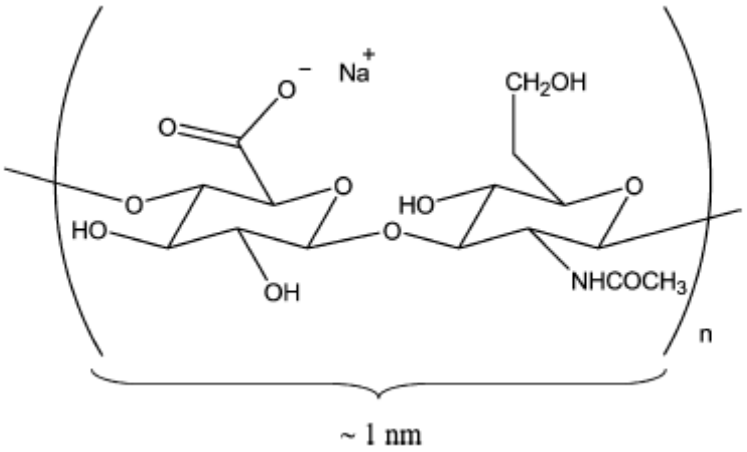

Fig. 1 *Structure of the disaccharide repeating unit of hyaluronic acid* [4].

When talked about hyaluronan, a natural biopolymer with a broad range of biomedical and industrial applications with unique viscoelastic and rheological properties is meant. These properties predispose HA to play an important role in living organisms and make it an attractive biomaterial for various medical applications.

2.1.1 Sources and Occurrence of HA

The purification and isolation of HA have been the centre of scientific interest for many decades [1]. HA is almost omnipresent in the human body and in other vertebrates [2], so it is hard to specify all its sources. The most important tissues are summarised in Tab. 1. The process of isolation to pure HA differs from its source. For example, Balazs first developed the extraction method for the isolation and purification of pharmaceutical grade HA [1]. As a

source he used umbilical cords and rooster combs which were frozen to destroy the cell membranes, and then HA was extracted with water and precipitated in organic solvents (ethanol, chloroform, etc.). After purification of the extract, 0.5 % protein impurities remained, the yield was 0.9 grams of HA per kilogram of the original material [1].

In the last decades, the bacterial production of HA by *Streptococcus equi* and *Streptococcus zooepidemicus* has been spread. This method brings larger yields that those achieved with the extraction methods. HA produced by *S. equi* has a lower MW than does the HA produced by *S. zooepidemicus,* which has MW of about 1.8 to 2 $\cdot 10^6$ Da. The yield is around 4 grams of HA per liter of the cultivated solution [1].

It is obvious that HA from various sources, with different degrees of purity and MW, is available for various medical application. The main impurities, depending on the source and isolation methods, are bacterial endotoxines, chondroitin sulfates, protein, nucleic acids, sodium chloride and heavy metals. Water is usually present in the range between 5 and 10 % in the very hygroscopic powder or fibrous aggregate [1].

Tab. 1 *Occurence of HA in different animal tissues and its content* [2].

Tissue or body fluid	Concentration (μg/ml)	Remarks
Rooster comb	7500	The animal tissue with by far the highest HA content
Human umbilical cord	4100	Contains primarily HA with a relatively high molar mass
Human joint (synovial) fluid	1400–3600	The volume of the synovial fluid increases under inflammatory conditions. This leads to a decreased HA concentration
Bovine nasal cartilage	1200	Often used as a cartilage model in experimental studies.
Human vitreous body	140–340	HA concentration increases upon the maturation of this tissue
Human dermis	200–500	Suggested as a "rejuvenating" agent in cosmetic dermatology
Human epidermis	100	HA concentration is much higher around the cells that synthesize HA
Rabbit brain	65	HA is supposed to reduce the probability of occurrence of brain tumors
Rabbit heart	27	HA is a major constituent in the pathological matrix that occludes the artery in coronary restenosis
Human thoracic lymph	0.2–50	The low molar mass of this HA is explained by the preferential uptake of the larger molecules by the liver endothelial cells
Human urine	0.1–0.3	Urine is also an important source of hyaluronidase
Human serum	0.01–0.1	HA concentrations increase in serum from elderly people as well as in patients with rheumatoid arthritis and liver cirrhosis

2.1.2 HA in Human Body

The highest amounts of HA in the human body and vertebrates are found in synovial fluid (Tab. 1) and extracellular matrix of soft connective tissues, although recently it has been shown to be present also intracellular (Evanko and Wight 2001, [2]). Other occurrence is in the umbilical cords and vitreous humour of the eye. Almost half of human body's HA occurs in skin with most of the HA located in the intracellular space. It may reaches the concentration 2.5 g l^{-1}. HA plays a series of important functions in skin. It can immobilize water in tissue and thereby change compressibility and dermal volume. It can also influence cell proliferation, differentiation, and tissue repair.

2.2 Biological Sources of Commercially Used HA

As it has been shown above (Tab. 1), HA is an essential functional component of almost all tissues in the vertebrate organism. Thus, various animal tissues – e.g. rooster combs, shark skin, bovine eyeballs – have been used as sources of isolation and production of high molar mass HA. Several separation procedures such as proteasedigestion, HA ion-pair precipitation, membrane ultrafiltration, HA non-solvent precipitation and/or liophylization have to be applied in order to obtain a pure compound. The average molar mass of the commercially available "extractive" HA preparations obtained from animal tissues is mostly in the range from several hundred thousands Da up to approximately 2.5 MDa. To date, the demand for HA materials approved for applications in human medicine has been satisfied by high molar mass HAs prepared from rooster combs.

Although animal tissues, primarily rooster combs, were involved at the early stages of production of the clinically utilizable materials. HA secreted by microorganisms such as certain attenuated strains of *S. zooepidermicus*, *S. equi* etc. is offered by many companies. The yield reaches several tons per year. Some of these "fermentative" HA preparations meet the demand on molar mass in the range of several MDa. However, the risk of mutation of the bacterial strains, possible co-production of various toxins, pyregens, immunogens, etc. hamper the broader application of fermentative HA in clinical practice. This is also the reason why HA samples originating from rooster combs are still currently prefered for human treatment in cases when HA material is designated for injection, e.g., in the eye, knee joint, etc. [2].

Thus at present, alternative sources for production of HA are being sought. One of the potential candidates is a genetically-modified bacterial strain, Bacillus subtilis. Such an engineered strain was able to produce HA with the molar mass in the 1 MDa range. The advantage of using *B. subtilis* is that it is easily cultivatable on a large scale and does not produce exo- or endotoxins. Moreover, *B. subtilis* does not produce hyaluronidase that could degrade the synthesized HA [1].

In the skin HA plays a role of a scavenger for free radicals generated by the ultraviolet rays from the sunlight. The ultraviolet light inflicts oxidative stress on cells and may damage their genetic material, thus causing degeneration and death of the cells.

In cartilage, despite its low content, HA acts as an important structural element of the matrix, forming an aggregation centre for aggrecan, a large chondroitin sulphate proteoglycan, that retains its macromolecular assembly in the matrix due to the specific HA-protein interactions.

In synovial fluid the high concentration of high MW HA provides necessary lubrication for the joint and serves as a shock absorber, reducing friction of the moving bones and diminishing wear of the joint.

HA is now recognized to play important roles in embryogenesis, signal transduction and cell motility, and is associated with cancer invasives and metastasis. Despite their uniform and simple primary structure, HA polymers have extraordinary wide-ranging and often opposing biological functions depending on the size of the molecule [2].

2.3 Biomedical Applications of HA

The usage of HA is very wide. It can be applied in a lot of biochemical and biomedical branches. Balasz classified basic areas of clinical applications of HA and its derivatives in 2004 [2] as follows :

- viscosurgery (in ophthalmological surgeries)
- viscoaugmentation (to fill and augment tissue space in skin, sphincter muscles)
- viscoseparation (to separate connective tissue surfaces traumatized by injury)
- viscosupplementation (to replace tissue fluids, such as replacement of synovial fluid in painful arthritis and to relieve pain)
- viscoprotection (to protect healthy, wounded or injured tissue surfaces from dryness or noxious environmental agents, and to promote healing of such surfaces)
- plastic surgery (to fill facial wrinkles and depressed scars)
- cosmetic industry (as an ingredient to some moisturing creams, balms)
- tissue engineering (tissue replacements, blood vessels, etc.)
- drug delivery systems, scaffolds, etc.

2.3.1 HA as a Drug Delivery System

As described in work [3], HA is responsible for various functions within extracellular matrix such as cell growth, differentiation, migration etc. A wide range of these activities can be explained by a large number of HA–binding receptors: cell surface glycoprotein CD44; receptor for HA–mediated motility RHAMM; several other receptors possessing HA–binding motifs, e.g. transmembrane protein LAYLIN; HA receptor for endocytosis (HARE);

lymphatic vessel endocytic LYVE–1; intracellular HA–binding protein including CDC37. HA is hence a useful polymer when talked about all tumor–targeting systems because the world–wide spread killing cancer.

Tumor cells overexpress many tumor–specific receptors, which can be used as targets to deliver cytotoxic agents into tumors. One of the tumor–targeting conjugates bearing cytotoxic agents, according to the type of cancer recognition moieties, is HA [5].

The higher concentration of HA in cancer cells is believed to form a less dense matrix, thus enhancing the cell's motility as well as invasive ability into other tissues and providing an immunoprotective coat to cancer cells. It is well known that various tumors, e.g. epithelial, ovarian, colon, stomach or leukemia, overexpress HA–binding receptors CD44 and RHAMM. Consequently, these tumor cells show enhanced binding and internalization of HA [3].

2.3.1.1 HA and Receptor CD44

Receptor CD44 is widely distributed cell surface glycoprotein and is particularly important in epidermal differentiation [5]. It has been reported [3] that the CD44 receptor contains the specific domain for HA, which consists of 160 amino acids residues. The binding affinity of CD44 to HA was found to be dependent on the size of HA oligomers. The high tumor specifity of the HA–CD44 interactions and high biocompatibility of HA were key factors for the design and synthesis of tumor–targeting bioconjugates bearing HA and cytotoxic agents.

Interactions of HA and CD44 play important roles in mediation or promotion of macrophage aggregation, cell migration, chondrocyte pericellular matrix assembly and leukocyte activation. It's known that the overexpress of HA synthases increases the HA level, which leads to the acceleration of tumor growth and metastasis.

2.3.1.2 HA and Receptor RHAMM

This type of receptors was discovered by Eva Turley [5]. RHAMM is an unusual protein, which enhances the motility of cells. It belongs to a heterogeneous group of proteins designated hyaladherins by their common ability to bind hyaluronan.

2.4 Distribution of HA According MW

HA as a polymer can be found and syntetized with different MW which determine its function in organism. *High molecular weight HA* (HMW HA) has MW more than 1000 kDa. It is used in medicine in wound healing because of its high affinity to water. It hydrates tissues and supports diffusion of ionts and nutrition. This type of HA plays an important role in vitreous humour and umbilical cord as a lubricant. *Low molecular weight HA* (LMW HA) has MW between 200 – 1000 kDa, protects skin against UV–radiation so it is used in several cosmetic products. It penetrates through the skin easily therefore it is used as a drug delivery system for biological active substances, cytostatics, etc.

Other group of polysaccharides is called *very low molecular weigh HA* (VLMW HA) and has MW between 10 – 200 kDa. Such a polymer supports production of melanin and is useful for diabetic wound healing. Finally, the last group of HA is formed by *oligosaccharides of HA* with MW lower than 10 kDa. Interactions of oligosaccharides are fully influenced by its length and target cell in organism.

2.5 Hydrophobically Modified Polymers

In general, hydrophobically modified polymers (HM polymers) are amphiphilic macromolecules, soluble in water, mainly constituted of a hydrophilic backbone and hydrophobic side groups [6]. The side groups are usually alkyl chains with different length. The physicochemical properties of HM polymers depend also on the structural parameters of the polymer, on the nature of the macromolecular backbone and environmental parameters (pH, salinity, temperature, etc.).
Landoll in 1982 [6] first reported the viscosimetric and surface–active properties of a series of nonionic polysaccharides derivatives with different hydrophilic backbones (methylcellulose, hydroxyethylcellulose and hydroxypropylcellulose). Since then other biopolymers have been hydrophobically modified: cyrboxymethylcellulose, dextran, pollulan, alginate, chitosan an hyaluronan [6]. Due to the amphiphilic character, HM polymers have high surface and interfacial properties. they diffuse through the bulk phase and adsorb at the interface, inducing the surface or interfacial tension of a polymer solution. The adsorption of at the air–water interface strongly depends on the macromolecular architecture, such as stiffness of the backbones and the charge density of the solvent – polyelectrolyte [6].

Other studies of surface properties of hyaluronan in dimeric surfactant solution indicated that the physical properties (critical micelle concentration, area per surfactant) strongly depend on the number of carbons in surfactant chain [7].

2.5.1. Modification of HA

To present, alkyl derivatives were prepared mostly in the form of carbamates or esters. The alkyl carbamates were prepared by the reaction of HA with alkyl amines using cyanogen bromide activation method. One of the characteristic parameter of any HA derivative is a degree of substitution (DS). It is defined as a molar ration of substituent and dimer unit of HA. This means that one alkyl substituent per one dimer unit represents a reference value (100 %) [8].

The methods of preparation alkyl esters is described in work [8]. The esterification of carboxylgroups of HA with therapeutically important alcohols produces water insoluble biopolymers with physical properties that are significantly different from HA itself. There are several possibilities for preparation esters. One of them is the method based on the reaction of HA with palmitoyl chloride in DMF in the presence of pyridine. Other methods of hydrophobization are based on the reaction of hydroxyl groups of glycosaminoglycan with fatty acid in trifluoroacetic acid anhydride or acetylation with acetanhydride in the presence of acetic acid and sulphuric acid.
The esterification, as mentioned in [8], was performed at alkaline pH with excess of 2-alkyloxymethyloxirane where alkyl was butyl, hexyl, octyl, decyl or tetradecyl. DS, which gives information about the structure of the prepared derivatives, was defined by NMR measurements

2.6 Surfactants and Micelles

2.6.1 Surfactant and Critical Micelle Concentration

Surface active substances, also called surfactants or tensides, consist of a polar head group and a long hydrophobic tail connected to the head group. The surfactants influence the surface properties of its solution – they lower the surface tension, more or less evidently. It is the common property of any surfactant.

When a surfactant is added to a water solution the surface tension decreases till some concentration of a solution. During this process the molecules of a surfactant diffuse in the solution and perform a layer at the surface. After addition of certain amount of the surfactant there is no more area free for other molecules of the surfactant and the surface of the solution is saturated. At this point the solution reaches its critical micelle concentration (CMC).

In other words, CMC is defined as the concentration of surfactants above which micelles are spontaneously formed [8]. Above CMC the values of the surface tension should not change any more (Fig. 2).

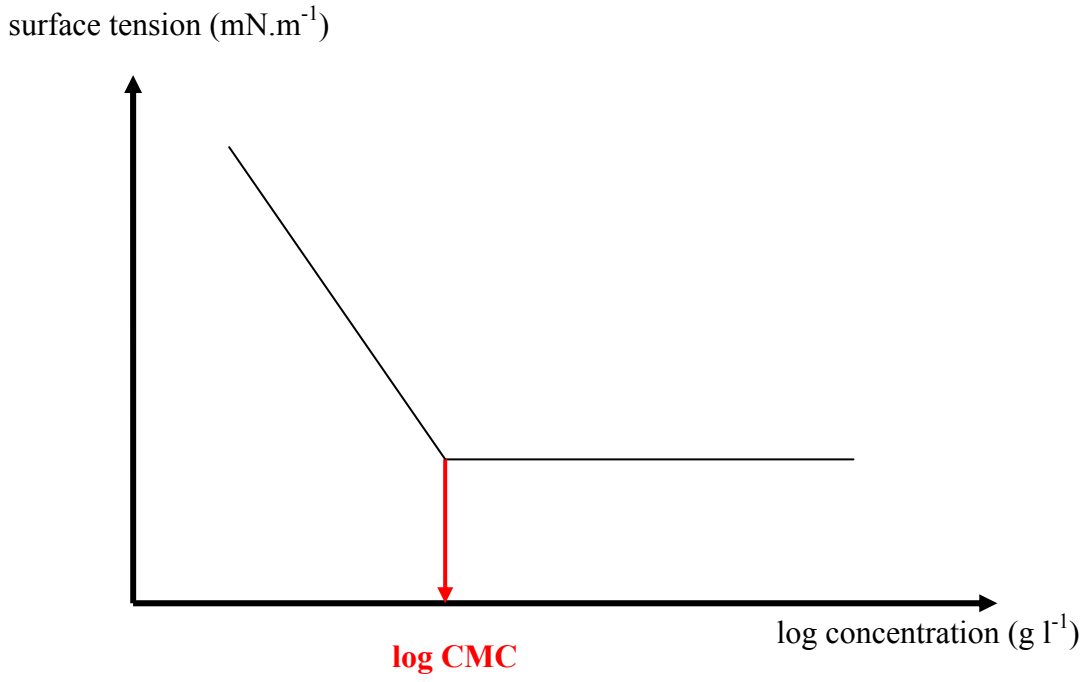

Fig. 2 *Schematic dependency of measured surfactant surface tension plotted vs. logarithm of concentration. The inflection point corresponds to the CMC. CMC is determined from the linear regression equations obtained after extrapolation of the two linear parts of the curve.*

The surface active properties of a surfacant provide the basis for various applications and are usually characterized by the CMC, surface concentration at the air–water itnerface, surface area per molecule and water surface tension reduction efficiency and effectiveness. Water

surface tension as a function of surfactant concentration has been the most widely used method to determine these parameters [10].

There is a world–wide known surfactant sodium dodecyl sulfate (SDS), $C_{12}H_{25}NaO_4S$, Fig. 3, which is a typical example of the surface active substance. The surface tension measurement of SDS in the presence of organic and inorganic salts is well studied. The salts as electrolytes are known to affect aggregation behaviour of surfactants. Thus, the surface properties of SDS were studied in this thesis for comparing the results to a high molecular weight molecule of HA and its derivatives.

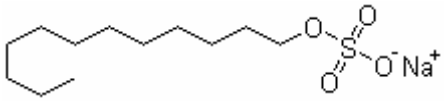

Fig. 3 *Sodium dodecyl sulphate formula* [12] .

Added electrolytes are known to affect the aggregation behaviour of surfactants. Surfactants can be devided into ionic and non–ionic. In case of ionic surfactants, the influence of added electrolytes on their micellization characteristics is attributed entirely to the counter ion effect [11]. Hence, this thesis is aimed at surface tension measurement of the derivatives of HA in different electrolyte media.

2.6.1.1 Critical Aggregation Concentration

As mentioned before, CMC is a characteristic parameter for a real surfactant, usually of low MW. In connection with hyaluronan and its solutions, including derivatives, another term is prefered, the critical aggregation concentration (CAC). The aggregate here means a nondefined cluster formed in the bulk solution. In the case of HA the specific shape and formation conditions are not uniquely defined, there is a very complicated system of inter- and intramolecular forces, which influence the final configuration of that clusters. These clusters may not consist of only one HA chain but also from many of them. Thus, at present paper CAC is used as a common term for determined concentrations which had to be measured by tensiometry and spectrophotometry.

2.6.2 Micelles, their Formation and Shapes

Micelles are the simpliest self–organising structures. They are characterized by CMC, aggregation number *N* or diffusion of micellar components. Aggregation number is defined as the number of molecules forming a micelle. Increasing the hydrocarbon chain length or adding salt lowers the CMC and increases *N*, while raising the temperature increases the CMC and lowers *N* [13].

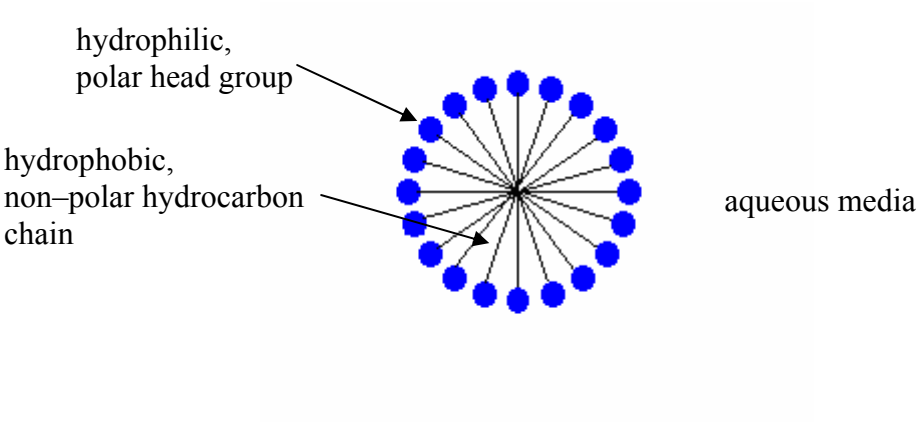

Fig. 4 *Structure of a micelle in aqueous solution* [14].

The process of aggregation happens above CMC. The micelle as type of an aggregate or a colloid particle formed at CMC is of various types: spherical, ellipsoidal, cylindrical, rodlike, disk–like micelle, membrane or vesicles. The precise shape of the micelle depends on the nature of the counterions [15]. These counterions can be $F^-, Cl^-, Br^-, Ac^-, NO_3^-$,etc. Counterions stabilize ionic surfactant micelles by binding to the micelles and screening the electrostatic repulsions and hence the binding affinity of the counterion influences the process of micellization. It follows that the counterion has a strong effect on the thermodynamics and aggregation properties of miccelization [16].

Micelles are continously disintegrating and reforming. They are in dynamic equilibrium with the surfactant monomers in bulk solution constantly being exchanged with the surfactant molecules in the micelles [17]. There are two relaxation times during the micellization process above CMC (Fig. 5).

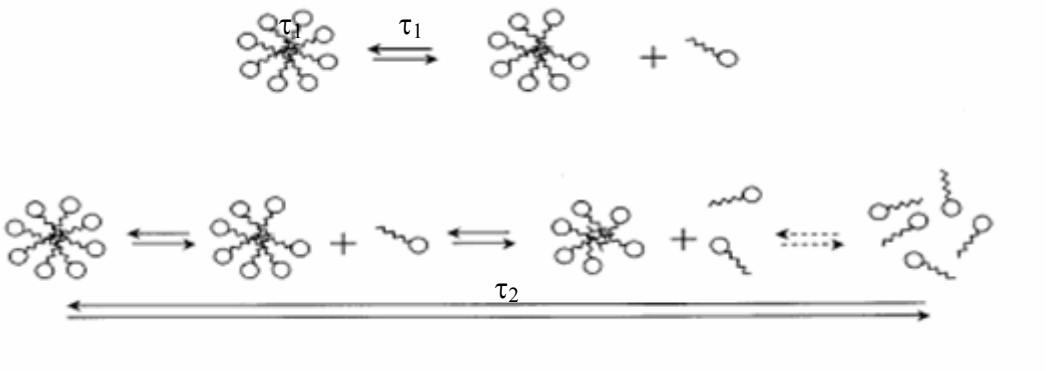

Fig. 5 *Schematic mechanism for the two relaxation times τ_1 and τ_2 for a surfactant solution above CMC* [17].

The relaxation time τ_1 is very fast, in microseconds. Then, slow relaxation process with time τ_2 (milliseconds to minutes) is followed. Thus, τ_2 represents the entire process of the formation or disintegration of micelles. It is also correlated with the average life–time of a micelle, and hence the molecular packing in the micelle, which in turn relates to the stability of a micelle. In conclusion, a long relaxation time τ_2 corresponds to a high dynamic surface tension [17].

2.6.3 Solubilization by Micelles

An important property of micelles that has a particular significance in field of drug delivery systems is their ability to increase the solubility of sparingly soluble substances. In aqueous system, nonpolar molecules will be adsorbed on the micelles surface, and the substances with intermediate polarity will be distributed along surfactants molecules in certain intermediate positions [18].

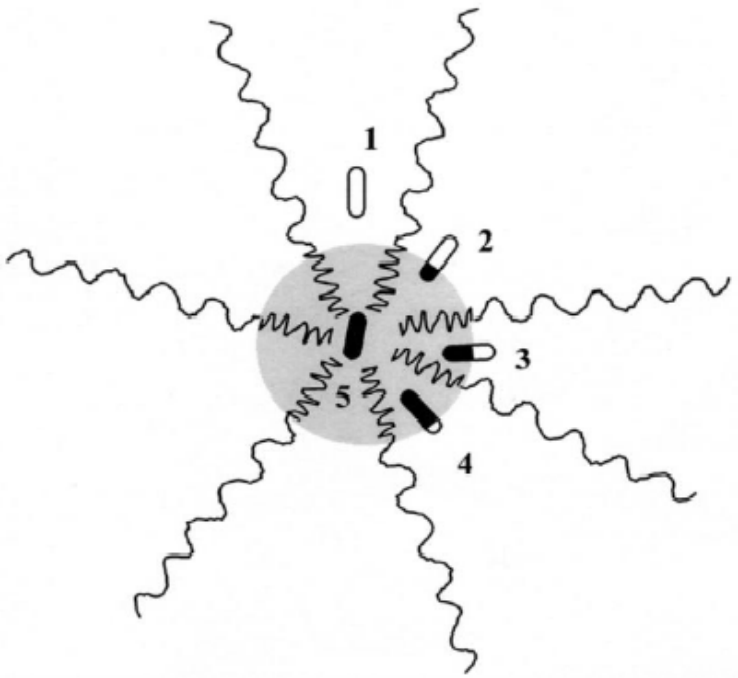

Fig. 6 *Possible paterns of drug association with a micelle depending on a micelle hydrophobicity* [18].

The five ovals in Fig. 6 symbolize a drug molecule, black colour shows the hydrophobic area, white shows the hydrophilic area. The oval number one is completely water-soluble hydrophilic drug, oval number five is completely insoluble hydrophobic drug. The ovals number two to four show drug molecules with intermediate hydrophobic/hydrophilic ratio and are located within the micelle particle.

In the work [19] behaviour of hydrophobically modified pullulan was studied by surface tension measurement and with a polarity probe the Coomassie Brilliant Blue dye (CBB).

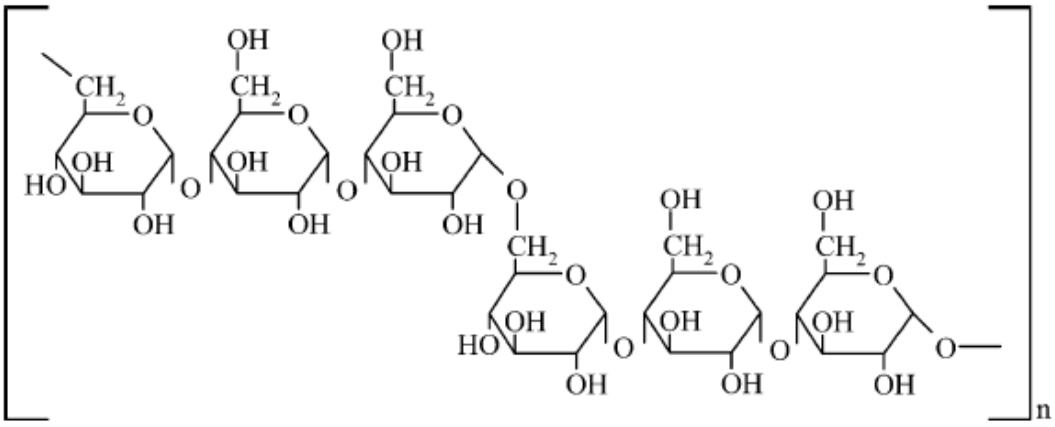

Fig. 7 *Structure of pullulan* [19].

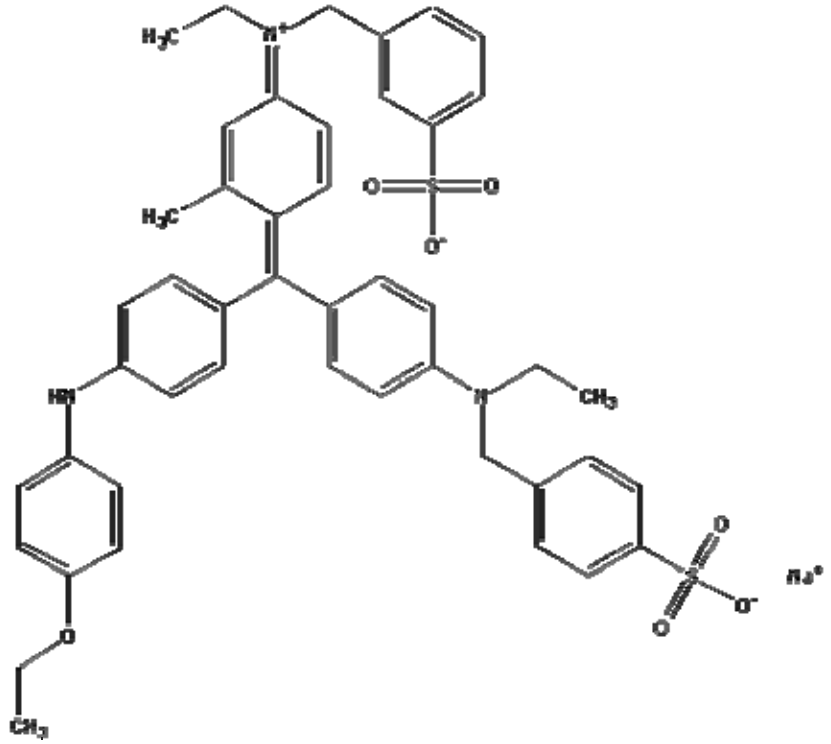

Fig. 8 *Structure of CBB* [20].

Polymeric surfactant exhibit higher micelle stability, due to various factors such as low critical concentration, slow exchange between free polymers and micelles, low mobility in the micelle core, higher molar mass, etc. So the hydrophobic microdomains in solutions of pullulan were evidenced with CBB. CAC was determined by plotting the absorption change at 618 nm versus logarithm of concentration. As expected, the absorption maxium of CBB is shifted (from 584 nm in polar media to 618 nm in apolar media) towards higher wavelengths when pullulan concentration increases, indicating that the environment of CBB becomes apolar. CBB thus constitutes a probe of its microenvironment polarity. The absorbance at 618 nm increases beyond critical polymer concentration.

To sum it up, the increase of absorbance at 618 nm in the case of hydrophobically modified samples is attributable to the presence of hydrophobic clusters and not to other kinds of interactions, such as electrostatic ones, that could occur between CBB and the polysaccharide. The hydrophobic clusters may result from intra- or intermolecular associations of hydrophobic chains on modified pullulan.
The comparing measurement of surface tension was performed using the Du Noüy ring method. CAC was obtained by plotting the results of surface tension versus logarithm of concentration. Using information from [19] experiments with derivatives of HA were performed. The second dye as a water-insoluble probe was chosen, Sudan III (Sudan Red BK, Sudan Red III).

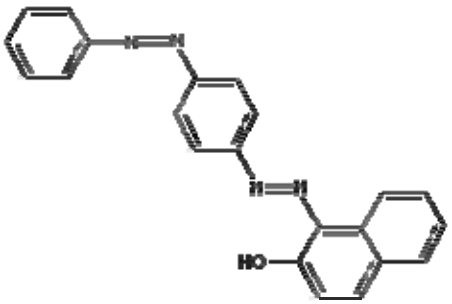

Fig. 9 *Formula of Sudan III* [21].

Sudan III is good soluble in organic solvents, such as ethanol, acetone or chloroform. In this case acetone was used to perform the spectrophotometric experiments. Thus, the solubilization of Sudan III in the samples prepared in water is observed just after a hydrophobic domain occurs in the bulk solution. And this happens above the CAC, which has to be find.

The principle how to determine CAC of solutions with HA derivatives and Sudan III is similar to the previous section with CBB. Maximum values of absorbance which was measured on a spectrophotometer was plotted versus logarithm of concentration. The intersection between two linear portions of the curve corresponds to CAC.

3 EXPERIMENTAL PART

3.1 Methods

The experimental part of this thesis has two parts – surface tension measurement and solubilization measurement.

3.1.1 Surface Tension

Generally, surface tension γ is defined as the magnitude of the force *F* exerted parallel to the surface of a liquid divided by the length *L* of the line over which the force acts:

$$\gamma = \frac{F}{L} \quad . \tag{1}$$

The unit of surface tension is N m^{-1}, force per unit of length.

Surface tension is a property of the surface of a liqiud which causes it to behave as an elastic sheet. It has the aim to reach the smooth surface with a minimum span. The surface requires the stage with a minimum of energy. So the surface of a liqiud will be smooth in every situation due to the minimum of the surface area. Surface tension allows small objects, such as insects, to keep at the surface of water. The chemical and physical behavior of liquids cannot be understood without taking surface tension into account [22].

Surface tension of liquids changes with their concentration. The influence of a solute on the surface tension of a solvent (γ_0) differs from the solute and the solvent. Both decreasing and increasing effect can be observed. Liquid are devided into three groups according to their effect to the change of the surface tension [23]:

- surface active or surfactants: decrease surface tension
- surface inactive: increase surface tension at higher concentration of the solute
- without any effect: the solute does not cause any change of surface tension (sugar in water)

Tensiometry is a technique about surface and interfacial properties of liquids. During surface tension measurement the cohesive energy, which is the result of attractive interactions among the molecules of the liquid, presents at a measured interface. The interactions of a molecule in the bulk of a liquid are balanced by an equal attractive force in all directions. Molecules at the surface of a liquid experience an imbalance of forces [24], (Fig. 10).

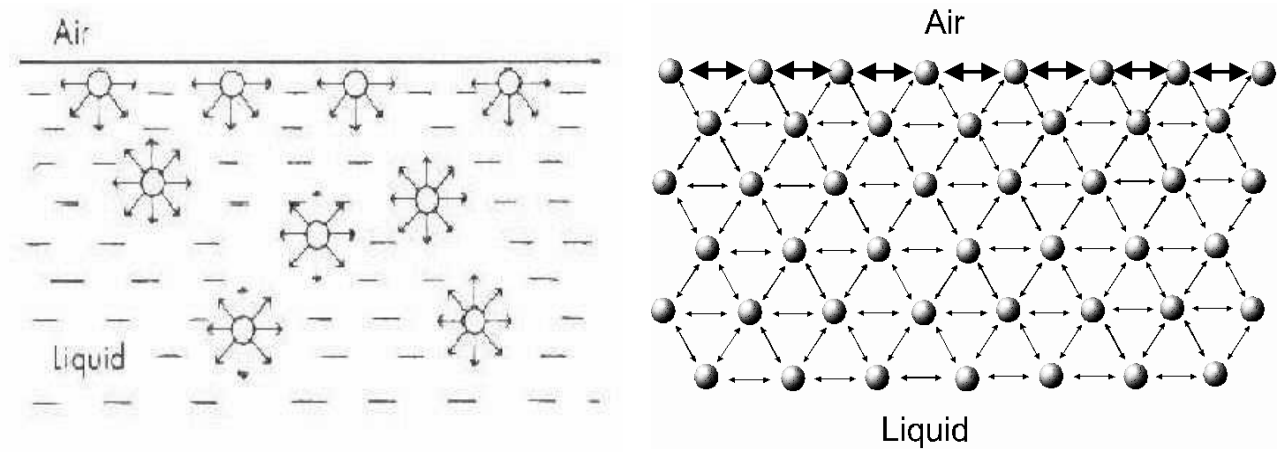

Fig. 10 *Intermolecular forces between molecules of a liquid* [25].

Polar liquids, such as water, have strong intermolecular interactions and thus high surface tension. Any factor which decreases the strength of this interaction will lower the value of surface tension. An increase of the temperature in the system, any contamination, especially by surfactants, will lower surface tension.

3.1.2 Surface Tension Measurement

Methods for determination of surface tension can be divided into three groups.

Static methods
Semistatic methods
Dynamic methods

Static methods are based on the observation of the stabilized state. Static method is, for example, a capillary rise method. Other possible method deals with shapes drops and bubbles. This method is called a pendant drop method. The most important of this group of methods is a Wilhelmy plate measurement method, which is described later. The aim of the semistatic methods is to reach the equilibrium state. The examples of these methods are stalagmometric method (measuring of the mean weight of the drop), the maximum bubble pressure method and the Du Noüy ring measurement method, which is trashed out later. Dynamic methods are used mainly for studying non-equilibrium states of liquids. Oscillating jet method belongs to this group of measuring methods.

3.1.2.1 Du Noüy Ring Measurement Method

Historically the ring method was the first to be developed. It is a method named after Pierre Lecomte du Noüy (1883 Paris – 1947 New York), a French biophysicist and philosopher [26]. The ring is made of Platinum and Iridium (usually 90 % Pt and 10 % Ir) [27]. In the ring method the liquid is raised until the contact with the surface is registered. The sample is then lowered again so that the liquid film produced beneath the ring is stretched. As the film is stretched a maximum force is experienced; this is recorded in the measurement [28].

Due to this method the maximum weight of the liquid lifted by a ring which is pulled out of a liquid surface or interface is measured. The force F required to lift the ring is related to the surface tension γ by the expression:

$$\gamma = aF \,, \tag{2}$$

where a is derived from the capillary pressure across the curved surface of the lifted liquid. The factor a depends on the geometrical dimensions of the ring and on the contact angle between the ring surface and the liquid. Reliable measurements can generally be made only if the ring is completely wetted by the liquid, i.e. contact angle equals to zero. Precise determination of the maximum force is achieved by repeatedly rising and lowering the ring close to the rupture of liquid lamella hanging from the ring. Just before the rupture of the lamella, force applied on the ring decreases dramatically. The balance detects this force change and lowers immediately the ring to eliminate lamella rupture [29].

Fig. 11 *Du Noüy ring* [29].

Surface tension is directly calculated by the instrument using the following equation:

$$\gamma = \frac{F}{4 \cdot \pi \cdot r_p} \cdot \Phi \,, \tag{3}$$

where F is the force needed for pulling the ring from the interface, r_p is the radius of the ring and Φ is the dimensionless correction factor and was obtained from the supplementary table of Huh and Mason [27]. For better understanding of the measuring process see Fig. 12.

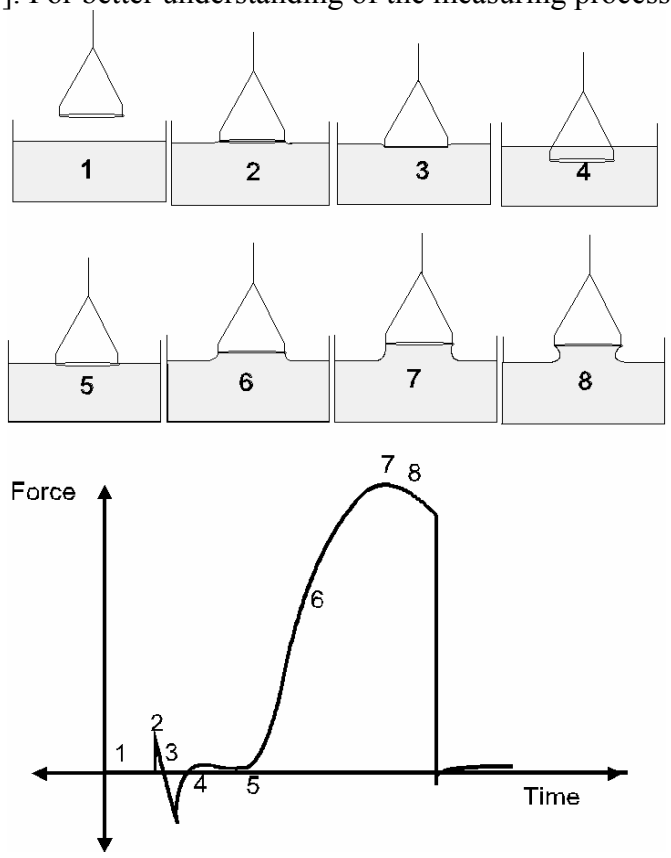

Fig. 12 *Schematic diagram of the ring method measurement* [29].

1. The ring is above the surface and the balance is zeroed.
2. The ring touches the surface and gets wetted resulting in some positive force.
3. Negative force is applied on the ring when it is pushed through the surface into the liquid.
4. Some positive force will remain because of the wetting of the vertical supports of the ring.
5. When pulled out of the liquid (by lowering the sample cup) the upper surface of the ring touches the liquid surface and the force starts to increase. This is because the surface tension of the liquid tries to prevent the ring from penetrating its surface.
6. The liquid is attached to the ring while pulling it up until the maximum force (7) is reached. At this point the volume of the liquid pulled up by the ring is also at its maximum causing the ring to detach from the liquid.
7. The maximum force needed to pull the ring from the liquid is proportional to the surface tension of the liquid. The greater is the force needed the greater is the surface tension.

3.1.2.2 Wilhelmy Plate Measurement Method

In this method, the weight of the liquid lifted when a plate is withdrawn through the surface is measured. This weight increases to some maximum value which, provided that the plate is completely wetted by the liquid and that the lower edge of the plate is at the same level as the flat surface, equals the surface tension times the length of the contact line between the plate and the liquid. In order to ensure the complete wetting the plate is usually made of platinum.

Surface tension is calculated according this equation:

$$\gamma = \frac{F_{\max} - F_V}{L_W \cdot \cos\theta}, \tag{4}$$

where γ is surface or interfacial tension, F_{max} is the maximum force, F_V is the weight of volume of liquid lifted, L_W is the wetted length and θ is the contact angle. The contact angle θ decreases as the extension increases and has the value 0° at the point of maximum force, this means that the term cos θ has the value 1.

There are two types of measurement by this method:

Static measurement
The sample surface is lifted until it contacts the lower edge of the plate. The wetting or capillary force pulls liquid up on the surface of the plate until equilibrium is reached.

Dynamic measurement
The plate is completely immersed into the liquid and then withdrawn through the surface until the maximum force is reached. In order to achieve a precise determination of the maximum the plate is not detached from the surface but repeatedly lowered and pulled back to the maximum [24].

The first idea, when the thesis was prepared, was to compare the results from the two methods, the ring and the plate one. But after some measurement were done, the results has shown that they are not comparable at all. It is because of the different process of the measurement. When talked about the ring measurement, the ring is hold at the surface of the sample for all the time of the measurement. It is only oscilating up and down at the surface.

Compared to the plate method, the plate is immersed and pulled up completely from the sample so the surface is being disturbed many times during one experiment. The Wilhelmy plate method is not suitable for polymer solutions. That's why all the experiments were performed only with the ring method.

3.1.3 Equipment for Tensiometry Measurement

The surface tension measurements were performed using the ring method. Measurements of surface tension were performed with a KSV Sigma 701 tensiometer. The diameter of a ring was 9.545 mm. The ring was flamed and immersed into diluted HCl before each measurement. The solution whose surface tension is to be measured was placed into the glass vessel which was boiled in distilled water.

Before measuring the samples water and solvent solution was measured every day. Measurement of one sample solution took approximately 40 minutes. One concentration serie was measured for the first time as a whole and then repeated two times more, so all the samples were measured three times. All the samples were measured at 25°C.

3.1.4 Solubilization Measurement

Solubilization of CBB and Sudan III in solutions of HA derivative was performed using Cary 50Probe UV–VISIBLE Spectrophotometer (Varian) and a quartz cell (1 cm) for spectrophotometry. As a blank solution water or NaCl stock solution was used in dependency on the samples.

All the samples were measured three times. When the samples with CBB were measured wavelength was set in the range of 500–700 nm, with Sudan III in the range of 600–400 nm. The speed of the scan was set to Medium adjustment for all the samples. It means 1 nm for one measured point.

First, the samples without a dye were measured, than the samples with the dye. The resulting data in form of absorbance of the dye were obtained as a difference between absorbance with a dye and without a dye. Finally, this data were used for determination of CAC.

3.2 Materials

3.2.1 Materials for Tensiometry

All used HA and its derivatives were obtained from CPN, spol. s.r.o., Dolní Dobrouč, Czech Republic as a white powder and wool, respectively. To prepare a stock solution the precisely weight HA in small amounts was added into water. The solutions were stirred for two days on a magnetic stirrer. After stirring them the solutions were stored in the fridge. The solutions were tempered at the room temperature before the measurement.

3.2.1.1 Samples of pure HA

Two types of HA were used for the measurement. First one has MW 0.46 MDa and the second one has MW 1.69 MDa. The specification follows:

Tab. 2 *Basic specifications of HA used for experiments.*

	HA 0.46 MDa	**HA 1.69 MDa**
dry matter	93.8 %	97.2 %
proteins	0.072 %	0.066 %
uronic acid	46.3 %	46.9 %
sodium hyaluronate	95.7 %	96.9 %

All the solutions were prepared with Sterile Water for Injection, Fresenius, Italy.

The measurements were performed with concentration series of solutions of HA in water and in 0.15 M NaCl. The concentration of NaCl was chosen to have the ionic strength similar to physiological saline solution. The concentration series had 8 samples, the concentrations of HA were in the range from 0.1 g l^{-1} to 2 g l^{-1}. The total volume of the samples was 15 ml. The samples were prepared using fine pipette.

When prepared samples with salt, the concentration series were first prepared only in water to volume 7.5 ml, then stirred for two hours and after that added 7.5 ml of 0.3 M NaCl to every sample. The samples solubilized better with water only and the stock solution of electrolyte was diluted exactly to concentration 0.15 M.

3.2.1.2 Samples of HA Derivatives

Two HA derivatives with different DS were used for surface tension measurement.

Tab. 3 *Specifications of HA derivatives.*

	MW (kDa)	**DS (%)**
C8NHHA9	494.7	100.0
C8NHHA10	481.4	7.58

Two abbreviations C8NHHA9 and C8NHHA10 consist of three parts: C8 means octyl as a side group on the hyaluronic chain, NH means that the side octyls are bonded by a carbamate bond, HA9 and HA10 is a number of a derivative.

There was the same method for preparation the samples with salts as used with HA. The concentration of a stock solution of any salt was two times higher due to its further dilution.

Other chemicals – SDS and five chloride salts with different cations (NaCl, KCl, $CaCl_2$, $MgCl_2$, $ZnCl_2$) were obtained from commercial sources and were used as recieved without any further purification.

Detailed list of concentration series for tensiometry is summarized in Tab. 4.

Tab. 4 *List of samples for surface tension measurement.*

Sample	**Salt conc. (mol l^{-1})**	**Range of conc. (g l^{-1})**	**Number of samples**	**Volume per sample (ml)**
HA 0.46 MDa water		0.01 – 2	8	15
HA 0.46 MDa Na^+	0.15	0.01 – 2	8	15
HA 0.46 MDa water		0.01 – 2	8	20
HA 1.69 MDa Na^+	0.15	0.01 – 2	8	20
C8NHHA9 Na^+	0.15	0.01 – 5	11	20
C8NHHA9 K^+	0.15	0.005 – 1	10	20
C8NHHA9 Ca^{2+}	0.05	0.005 – 1	10	20
C8NHHA9 Mg^{2+}	0.05	0.005 – 1	10	20
C8NHHA9 Zn^{2+}	0.05	0.005 – 1	10	20
C8NHHA9 water		0.03 – 10	10	20
C8NHHA10 Na^+	0.15	0.01 – 4	14	20
SDS water		5×10^{-5} –5×10^{-2} *	11	20
SDS Na^+	0.15	5×10^{-5} –5×10^{-2} *	11	20

*Concentration series of SDS were prepared in concentrations in mol l^{-1}.

3.2.2 Materials for Solubilization Measurement

Hydrophobic microdomains in solutions of C8NHHA9 in water and NaCl were evidenced with CBB and Sudan III dyes (Fig. 8, Fig. 9). The samples were first used for surface tension measurement and then for solubilization.

The dyes were obtained from Fluka and were used as recieved. The concentrations of the stock solutions of the dyes were $2.55\ \mathrm{g\ l^{-1}}$ for CBB in water and 1.5×10^{-5} M for Sudan III in acetone. All the samples had 5 ml of total volume; the dose was 50 μl per sample of CBB and 100 μl of Sudan III. When prepared the samples into the plastic test tubes the dye was put first and then the sample before evaporating acetone from Sudan III. The samples were stirred in plastic tubes for 24 hours and they were centrifuged for 45 minutes under 4000 revolutions per minute before measurement. The measurement was performed at room temperature.

4 RESULTS AND DISCUSSION

4.1 Surface Tension Measurement

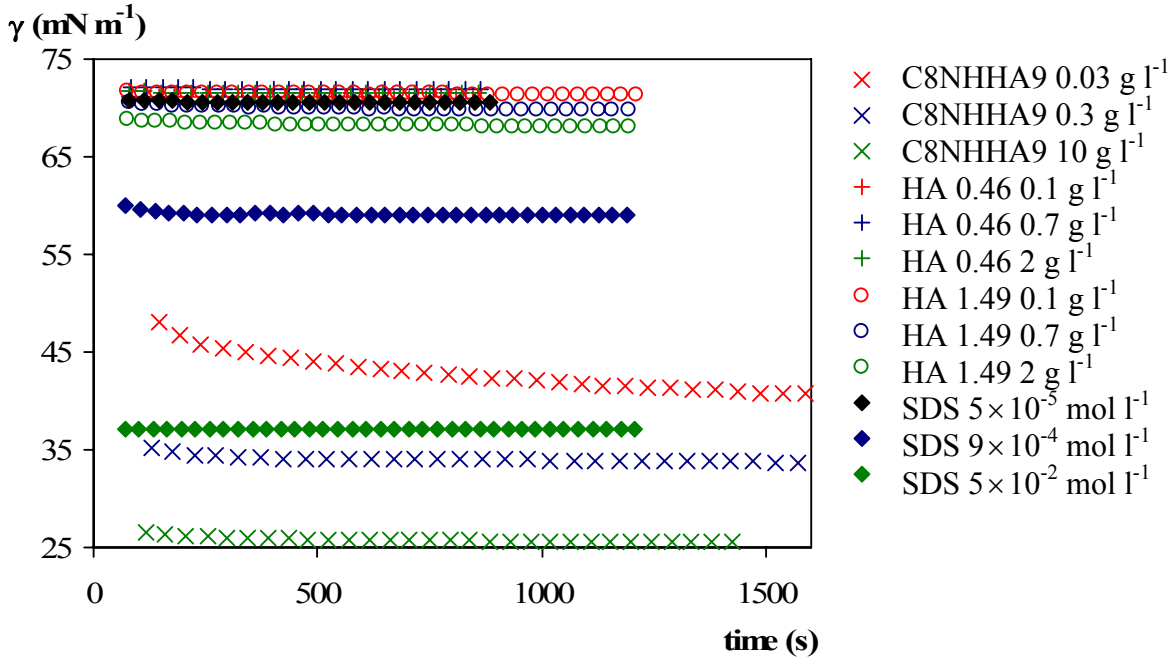

Fig. 13 *Stability of the measured samples in time.*

There is the stability of the derivatives, two HA and SDS (all in water) in time shown in Fig. 13. There are three concentrations of each substance chosen for illustration of different behaviour of the samples during the tensiometry measurement. There is an evidence of surface tension decrease of the derivative C8NHHA9, especially in the case of low concentration. SDS and both HA shows stable behaviour.

As Fig. 13 represents, stable values of surface tension of HA solutions surfactant behaviour are observed. The values are in the range from 71 to 72 mN m^{-1} for HA 0.46 MDa and from 68 to 72 mN m^{-1} for HA 1.49 MDa. On the other hand, as expected, SDS and derivatives of HA decrease surface tension.

The concentration of SDS is usually given in units mol l^{-1}, thus in Tab. 5 there are recalculated values to mass concentration in g l^{-1} to be good comparable with the rest of the data in Fig. 13.

Tab. 5 *Recalculated concentrations of SDS.*

SDS	
mol l^{-1}	g l^{-1}
5×10^{-5}	0.014
9×10^{-4}	0.026
5×10^{-2}	14.419

The stable value of surface tension was determined as the mean value from last 10 minutes of the measurement. The second possibility is to plot the surface tension data versus $\frac{1}{\sqrt{t}}$, where *t* is the total time of measurement, to get the extrapolated data for infinite time. These results are theoretical but can be compared with the real ones.

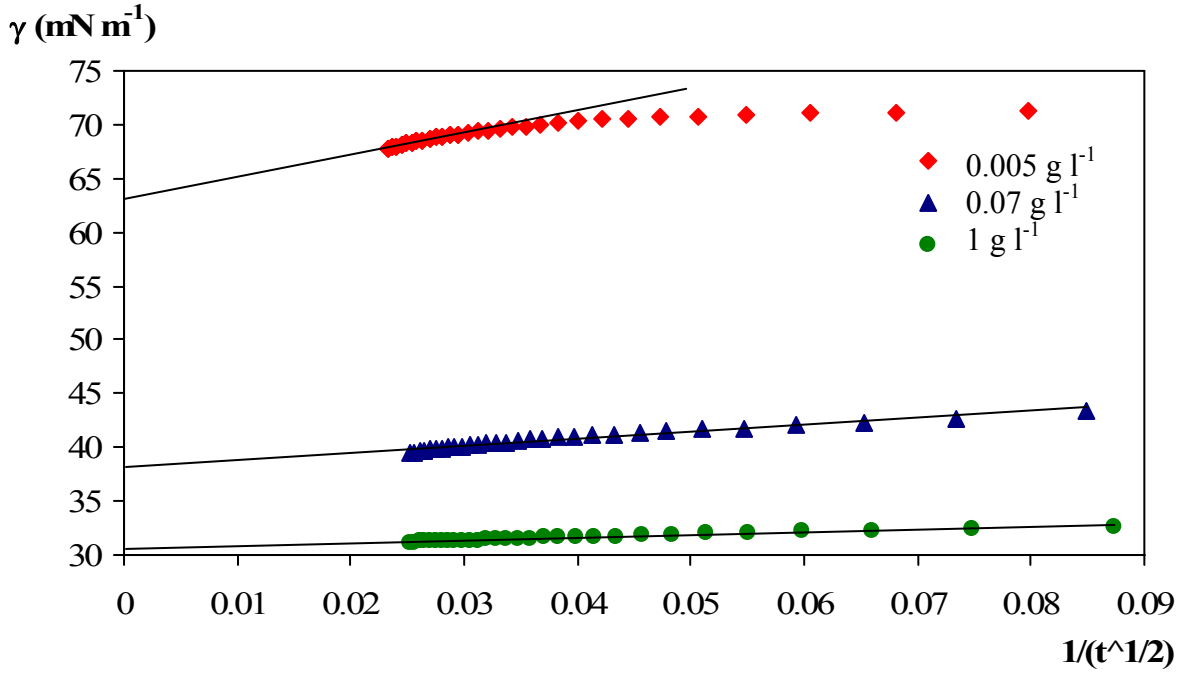

Fig. 14 *Results of surface tension of C8NHHA9 in 0.15 M KCl.*

The Fig. 14 represents the plot of surface tension vs. $\frac{1}{\sqrt{t}}$. There are three concentrations which show the decrease of surface tension in time and the black line symbolize the extrapolation (regression equation) to infinite time. The results for this solution, as an example, are given in Tab. 6.

Tab. 6 *Results of surface tension for solution of C8NHHA9 in 0.15 M KCl.*

Concentration	**Mean value of γ**	**Results from extrapolation**
(g l^{-1})	(mN m^{-1})	γ (mN m^{-1})
0.005	68.352	63.003
0.007	54.621	43.178
0.01	47.619	44.943
0.03	42.072	41.301
0.07	39.813	38.185
0.1	38.135	35.847
0.3	34.190	33.053
0.5	33.071	31.894
0.7	31.987	31.028
1	31.260	30.596

It shows that the mean values of surface tension obtained from the measured data are higher that the results from the extrapolation. The differences between them decrease with increasing concentration, because the surface of solutions with higher concentration is more and more stable in time, which causes lower decrease of the last few values.

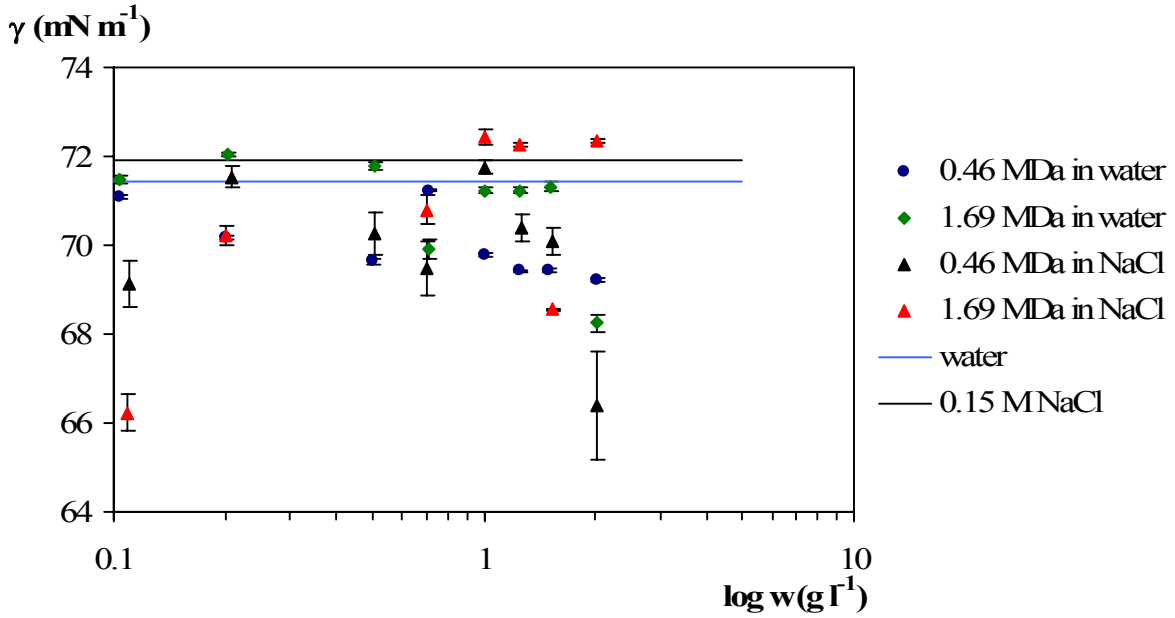

Fig. 15 *Compared results of surface tension of two HAs, both in water and in 0.15 M NaCl.*

In Fig. 15 surface tension of HA 0.46 MDa and 1.49 MDa in different media is plotted vs. logarithm of mass concentration. The two lines, for pure water and 0.15 M NaCl, are added. The influence of the ionic strength, which was 0.15, indicates the decrease of surface tension in case of HA 0.46 MDa more than HA 1.49 MDa. Generally, the data do not show the decrease of surface tension and surface active behaviour in both environment.

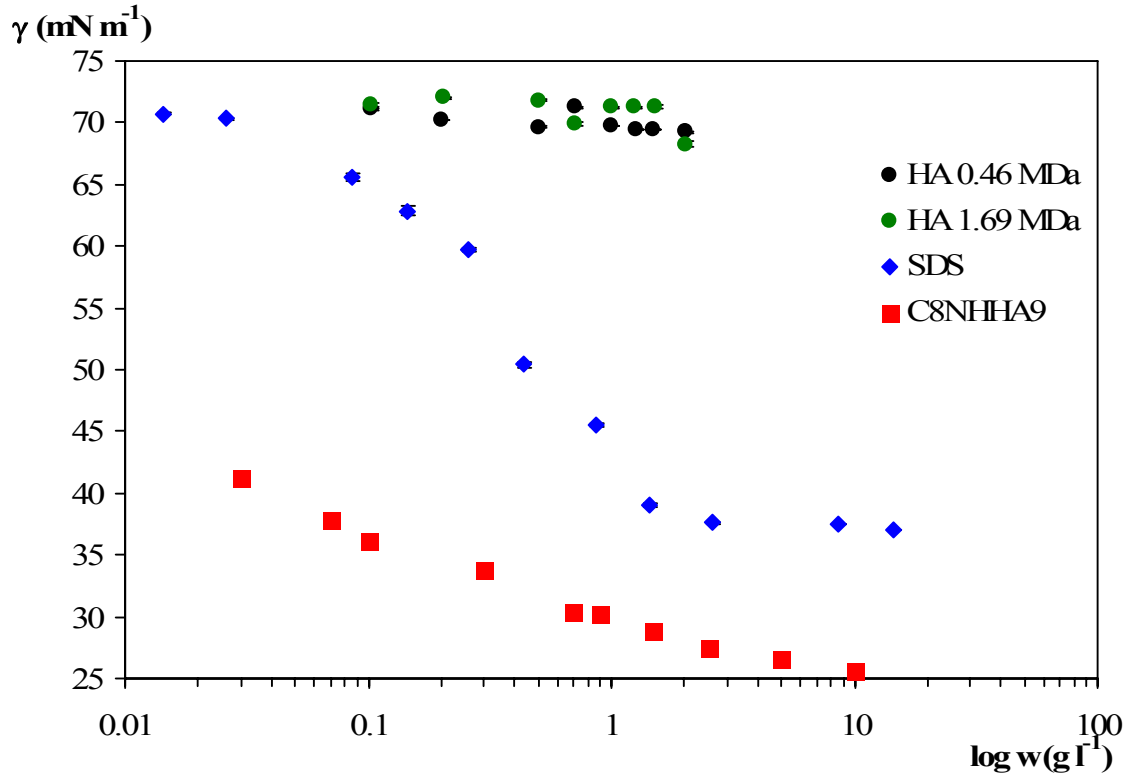

Fig. 16 *Surface tension of HAs, SDS and derivative C8NHHA9 in water.*

The data in Fig. 16 display the big differences in surface tension values of HA as a polymer, SDS as a low molecular substance and a hydrophobically modified HA in water environment. SDS, a typical example of a surfactant, shows a strong decrease of surface tension till it reaches CMC. It starts at about 70 mN m^{-1} and the last measured point has 37 mN m^{-1}.

The decreasing tendency is also visible on the curve of the derivative. The values are in the range of 41 to 26 mN m^{-1}. On the other hand, as written above, HA keep the stable values near 70 mN m^{-1}.

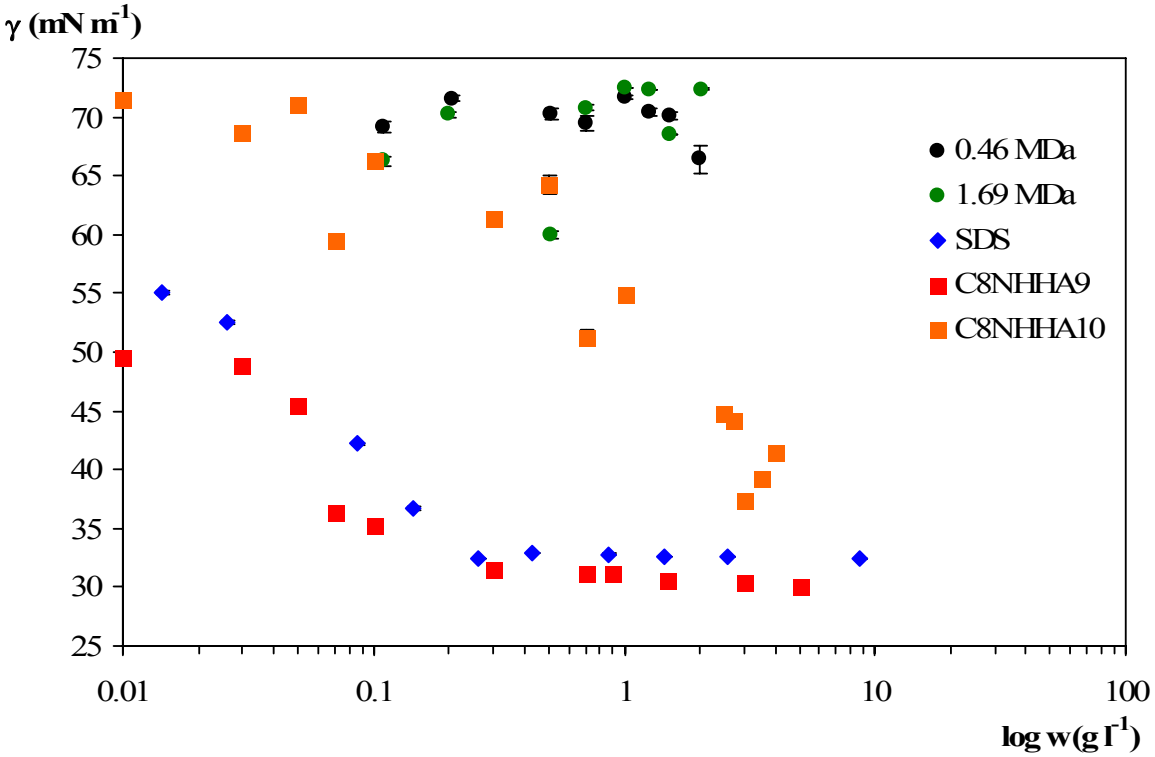

Fig. 17 *Surface tension of HA, SDS and derivatives C8NHHA9, C8NHHA10 in 0.15 M NaCl.*

The similar diagram represents the results in 0.15 M NaCl. There is not a big difference in case of HA. The initial value of surface tension of SDS is decreased form 70 mN m^{-1} in water to 55 mN m^{-1} in NaCl. The results of derivative C8NHHA9 in NaCl show a stronger decrease.

If the two derivatives are compared, C8NHHA10 indicates that the results do not decrease linearly and the inflection point is not observed. The DS of C8NHHA10 is around 10 %, which allows to make more intra- and intermolecular interactions and the surface film is being changed.

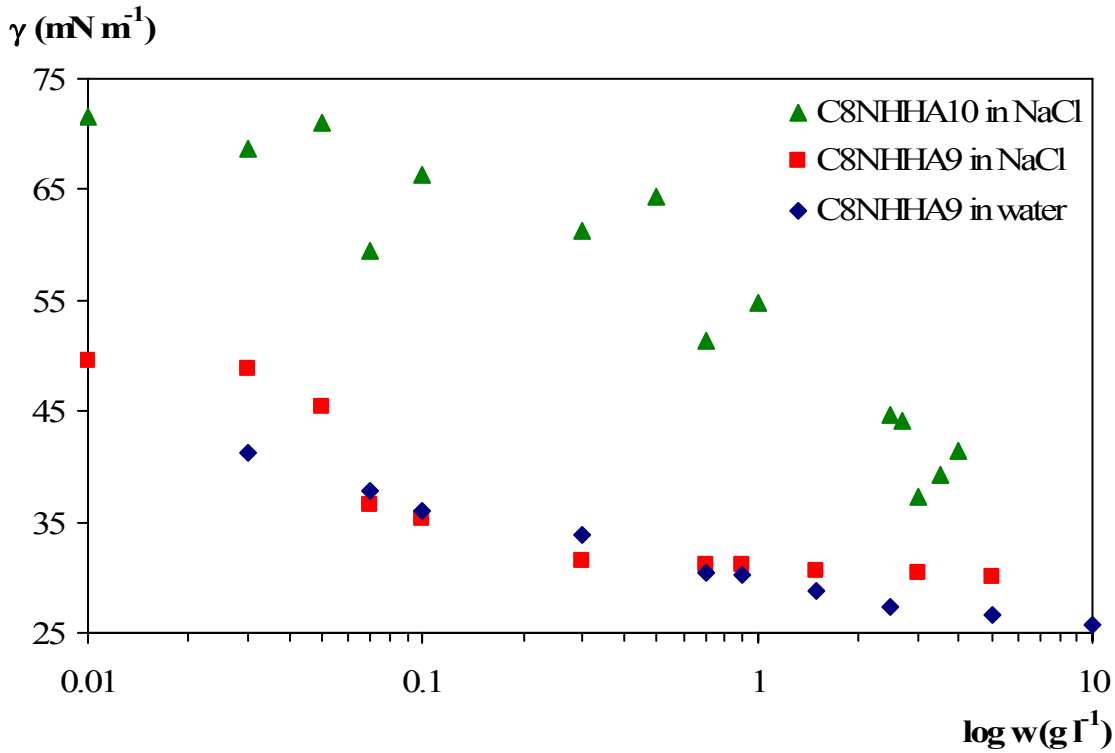

Fig. 18 *Influence of the ionic strength on the solutions of two derivatives.*

There is a difference between the behaviour of these derivatives varying not only in media, but also in degree of substitution. On the curve of the derivative C8NHHA9 in both environment we observe an intersection between two linear portions of the curve. This does not occur in case of C8NHHA10.

The derivative C8NHHA10 has higher surface tension because of smaller ratio of hydrocarbon chains. Thus, the polymer chain of the derivative can absorb more NaCl (dissolved in water), which increases the surface tension values. The linear section of the curve expected on the right part may be obtained at higher concentrations of the samples.

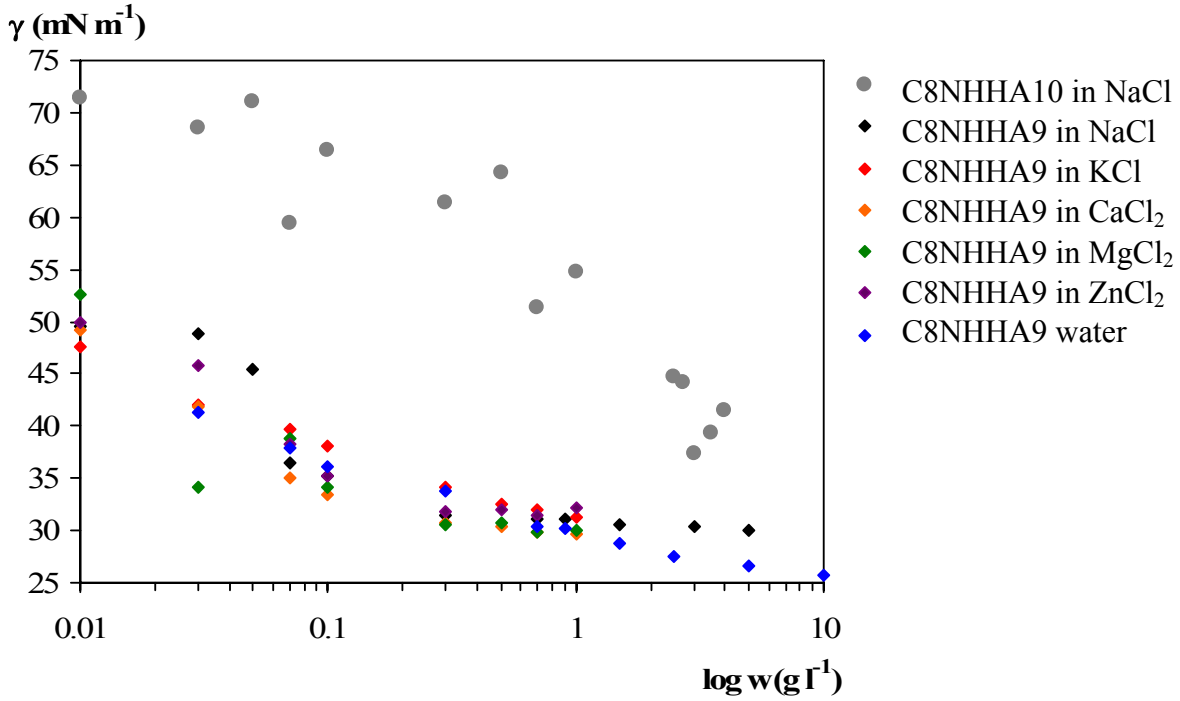

Fig. 19 *Influence of different ions on surface tension of HA derivatives.*

The influence of different ion environment is displayed in Fig. 19. The data are distributed in the broad range at low concentrations. Then, near CAC, the values are closer to each other. There is not a big difference between Ca^{2+} and Mg^{2+} ions around CAC. It is caused by their position in the periodic table of the elements. Some larger differences can be observed in case of water and Na^{+}. For better interpretation see Tab. 7.

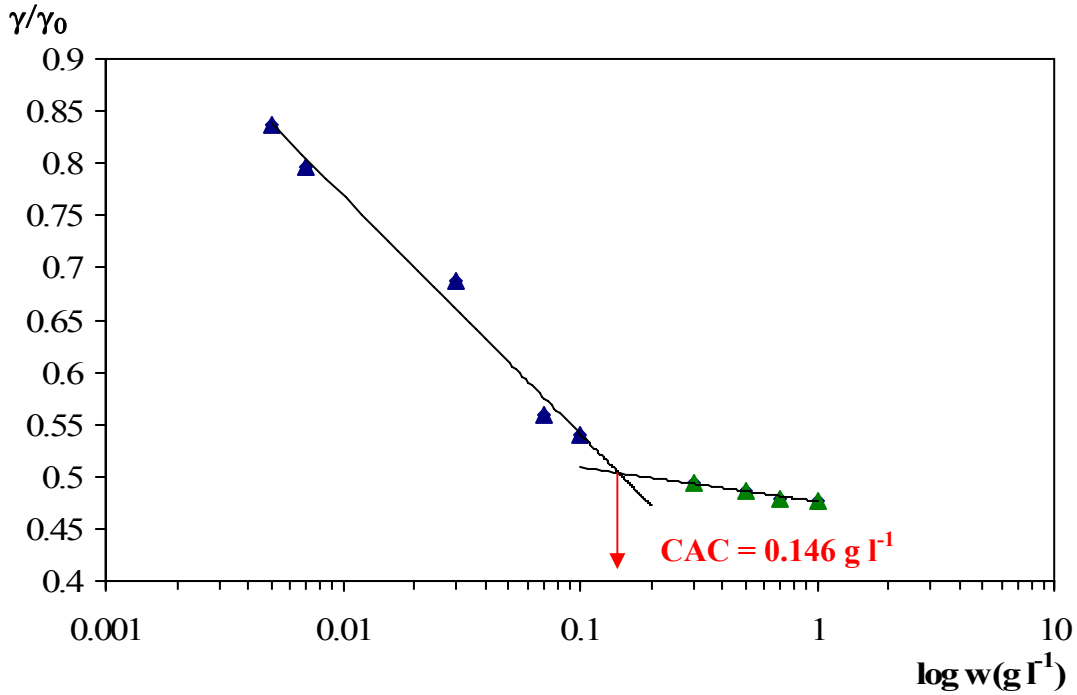

Fig. 20 *Relative values of surface tension of derivative C8NHHA9 in 0.05 M $CaCl_2$.*

This kind of diagram is used for determination of CAC. Several additional characteristic parameters including the maximum surface (excess) concentration of surfactant at the air–water interface Γ, the surface area occupied by each surfactant molecule A_m, the efficiency pC_{20} and the effectiveness (Π_{CAC}) of the reduction in surface tension can be obtained from surface tension plots and the simplified Gibbs adsorption equation [10]:

$$d\gamma = -2.303RT\,\Gamma\, d\log \mathrm{CAC}, \tag{5}$$

where γ (mN m^{-1}) is surface tension, R (J mol^{-1} K^{-1}) is gas constant, T is temperature (K) and Γ (mol m^{-2}) is maximum surface concentration.

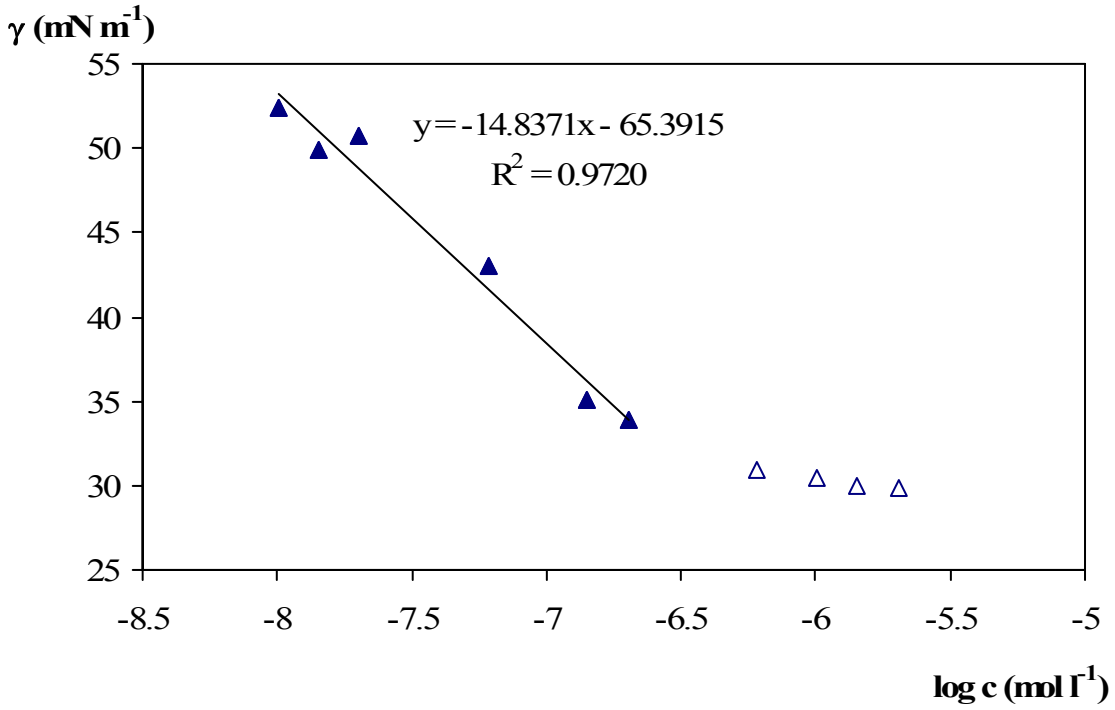

Fig. 21 *The diagram of surface tension of C8NHHA9 in 0.05 M $CACl_2$ in the suitable form for calculation other parameters.*

Maximum surface concentration Γ can be directly calculated from the slope of surface tension against log c (mol l^{-1}) plot, Fig. 21:

$$\Gamma = -\frac{k}{2.303RT}, \tag{6}$$

where k is the slope in N m^{-1}. The surface area A_m (m^2), but usually given as A_m ($Å^2$), occupied by each molecule is approximately $\frac{1}{\Gamma N_A}$, where N_A is Avogadro's number. The efficiency and the effectiveness describe the reduction in surface tension. The efficiency is defined by the value of the negative logarithm of the bulk concentration necessary to reduce the surface tension by 20 mN m^1:

$$pC_{20} = \frac{-[(\gamma_0 - 20) - K]}{k}, \tag{7}$$

where γ_0 is surface tension of the solvent and K is the intercept in the equation in Fig. 21.

The effectiveness, Π_{CAC}, is an important parameter in characterizing the reduction in water surface tension, because it does not always follows the same trends as the efficiency.
Effectiveness is defined as the extend of surface tension reduction attained at CAC:

$$\Pi_{CAC} = \gamma_0 - \gamma_{CAC}, \tag{8}$$

where γ_{CAC} (mN m^{-1}) is surface tension at CAC and is calculated from (9):

$$\gamma_{CAC} = k \log C_{CAC} + K, \quad (9)$$

where $\log C_{CAC}$ (mol l^{-1}) is the logarithm of CAC obtained from Fig. 20. The relative value of Π_{CAC} in percentage is obtained after dividing of Π_{CAC} by the surface tension of the solvent (γ_0). All the parameters calculated according to (6 – 9) are summarized in Tab. 7:

Tab. 7 *Summary of results from tensiometry.*

	CAC (g l^{-1})	**$\Gamma \times 10^{-6}$ (mol m^{-2})**	**pC20**	**Π_{CAC} (mN m^{-1})**	**Π_{CAC} (%)**	**A_m (Å^2)**
SDS water	1.671	3.92	2.81	32.98	46.18	42.34
SDS Na^+	0.245	3.31	2.74	38.96	54.19	50.16
C8NHHA9 water	1.800	1.30	8.65	45.90	64.27	127.44
C8NHHA9 Na^+	0.120	5.02	7.30	39.63	55.13	33.11
C8NHHA9 K^+	0.071	3.88	7.18	27.67	41.36	42.79
C8NHHA9 Mg^{2+}	0.034	7.37	7.55	32.03	47.30	22.54
C8NHHA9 Ca^{2+}	0.146	2.38	7.32	31.13	49.71	69.79
C8NHHA9 Zn^{2+}	0.221	3.46	7.51	40.06	56.02	48.05
C8NHHA10 Na^+	0.897	1.08	4.44	17.67	24.58	153.48

As expected, when CAC of these six types of derivative C8NHHA9 solutions is compared, there are several differencies between them. Water, which dissociates into small ions, increase the CAC. This applies for SDS solutions, too. The metal ions allow to aggregate at lower concentrations. CAC decreases with increasing possition of the ion in the periodic tabel in case of Na^+ and K^+. On the other hand, CAC increases in other three ions environment. It is probably caused by the size of ions and their divalency (2+). The question is, what configuration of aggregates the ions actually form.

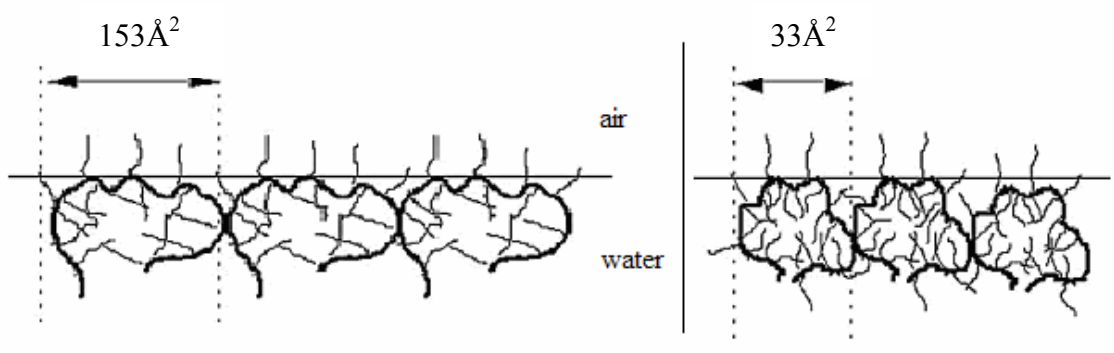

Fig. 22 *Role of different DS on the area occupied by surfactant molecules* [6].

C8NHHA10 has CAC almost five times higher than C8NHHA9, both in Na^+ environment. It is due to the lower DS, this means that there is more free space between derivative chains for ions and it needs more concentration to form aggregates. This is also the reason, why the areas per molecule A_m, summarized in the last column, differs a lot. It corresponds to [6], where the comparison of two hydrophobically modified pullulans with different DS are described, Fig. 22. When DS is lower it leads to the enlargement of the interfacial molecular area.

4.2 Solubilization Measurement

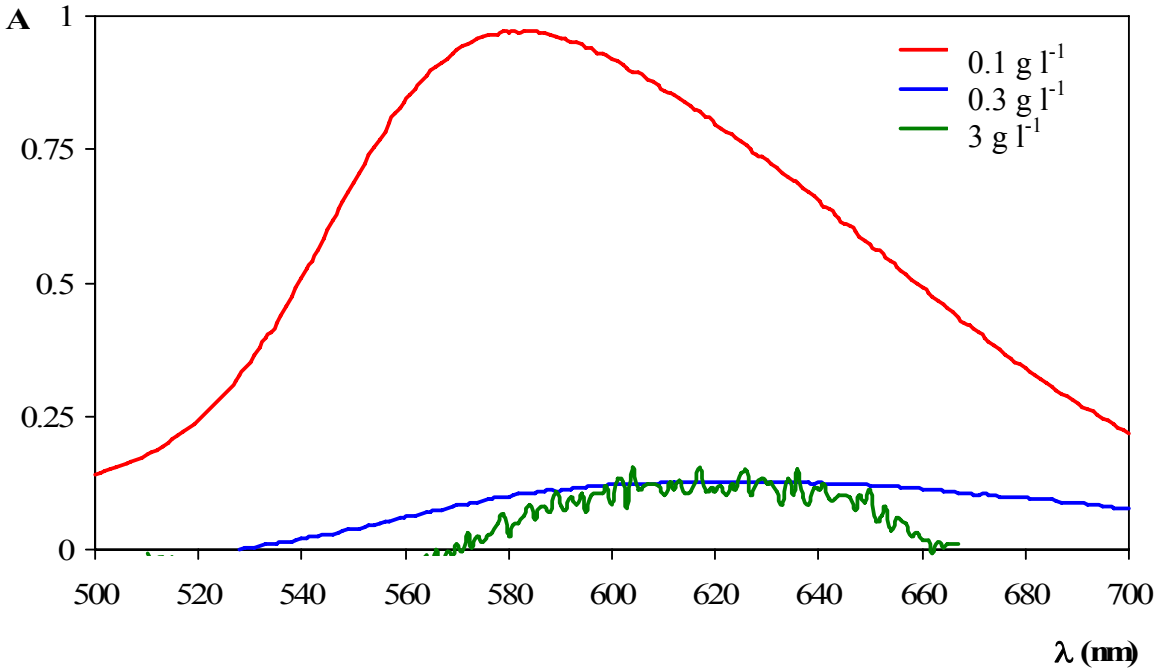

Fig. 23 *UV–VIS absorption spectrum of CBB in C8NHHA9 with 0.15 M NaCl.*

The absorption spectrum of for three concentrations 0.1 g l^{-1}, 0.3 g l^{-1} and 3 g l^{-1} of derivative C8NHHA9 in 0.15 M NaCl with CBB shows the shift of the absorption maximum to higher wavelengths as described in work [19]. The wavelength was set into the range of 500 to 700 nm. The wavelength of the maximum λ_{max} of the sample with 0.1 g l^{-1} is 585 nm and λ_{max} for 3 g l^{-1} is 617 nm. Thus we suggest that the environment changes its polarity from polar to apolar. It gives an evidence about forming some aggregates, so the concentration is beyond CAC.

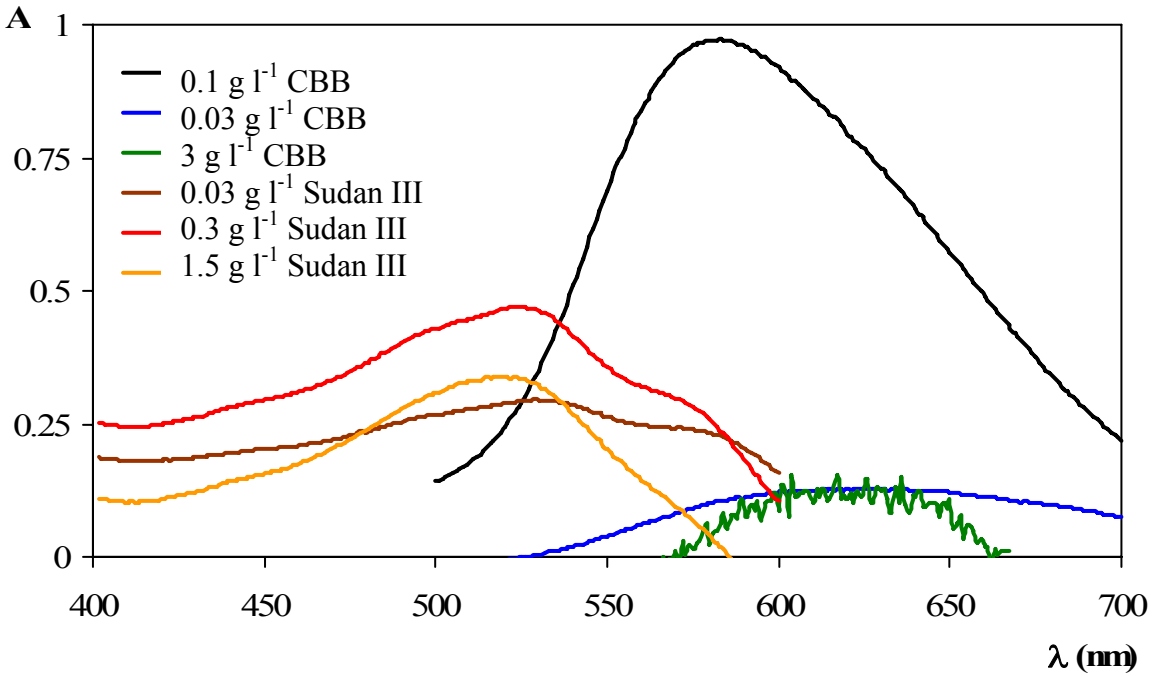

Fig. 24 *Comparison of absorption spectra of the two dyes in different media.*

The absorption spectra of CBB (C8NHHA9 in NaCl) and Sudan III (C8NHHA9 in water) at different concentration is shown in Fig. 24. The maximum absorption peak of Sudan III is found around 525 nm, the absorption maximum of CBB is found between 525 nm and 620 nm.

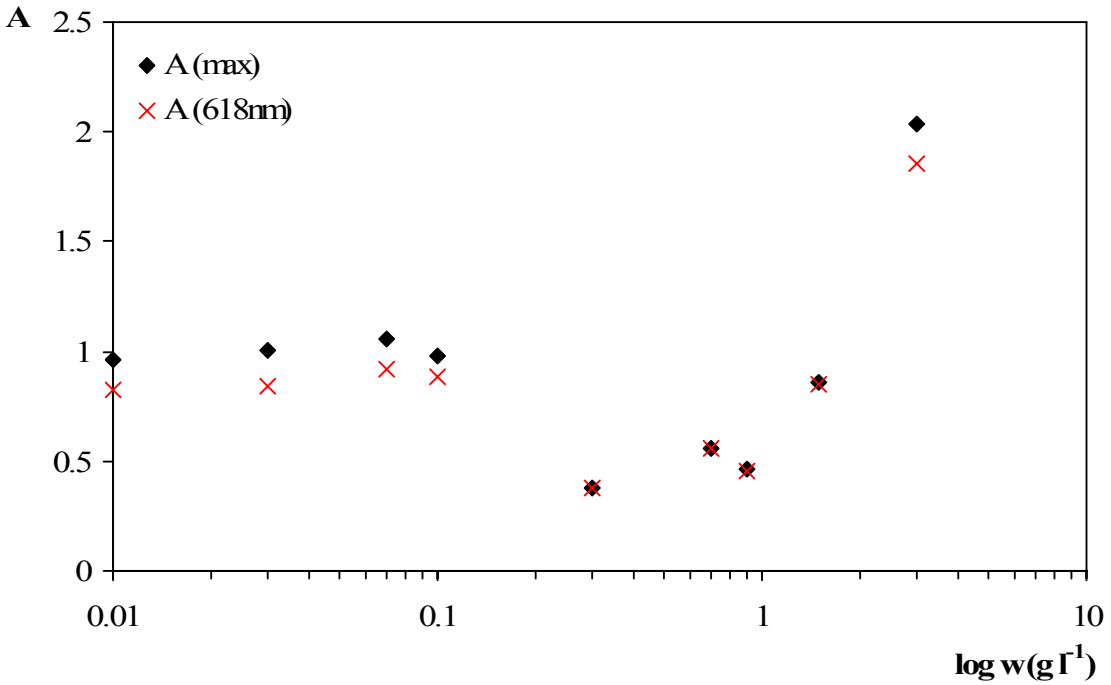

Fig. 25 *The change of absorption (C8NHHA9 in NaCl with CBB) at A_{max} and A_{618nm}.*

The Fig. 25 showns the change of absorption samples of derivative C9NHHA9 in NaCl in dependence on A_{max} and A_{618nm}. There is not a big difference, especially at higher concentrations. The results fit well. The value of CAC can be obtained as the concentration at the intersection point of the curve.

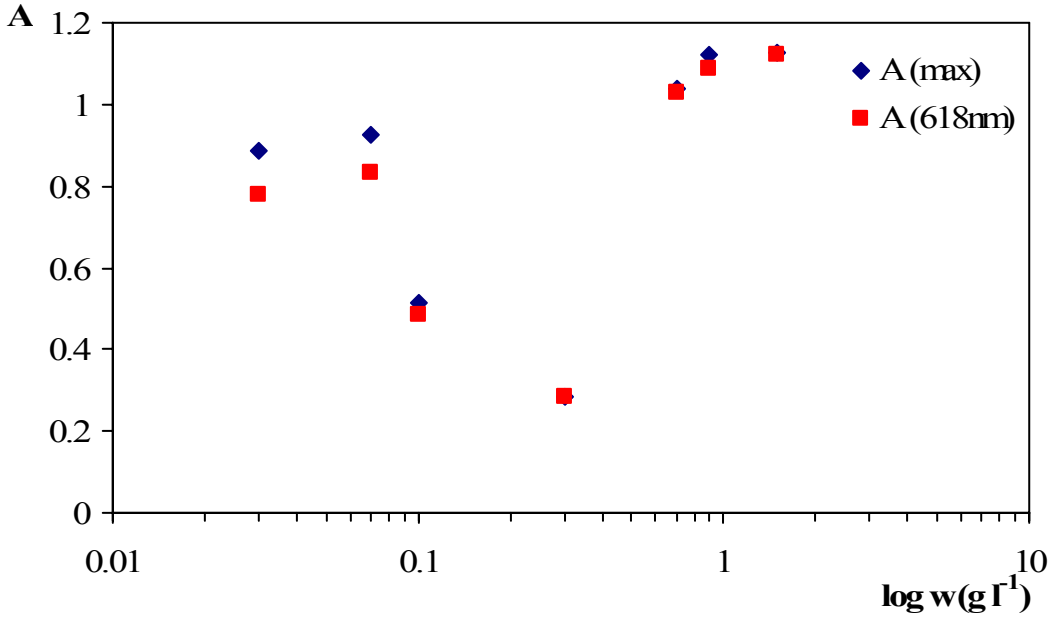

Fig. 26 *Behaviour of absorbance of CBB in C8NHHA9 in water environment.*

Results of absorbance of CBB vs. concentration of derivative C8NHHA9 in water is shown in Fig. 26. The decreasing section of data corresponds to the range of concentrations, where the hydrophobic domains are being formed. The next increasing is observed beyond CAC. As compared with [19], the increase of absorbance at A_{max}, resp. A_{618nm} in the case of hydrophobically modified samples is really attributable to the presence of hydrophobic clusters and not other kinds of interactions, such as electrostatic ones, that could occur between CBB and the polysaccharide.

In other words, CBB is a water soluble dye, so if absorbance decreases, some CBB molecules are not dissolved and it causes lower detected value of absorbance.

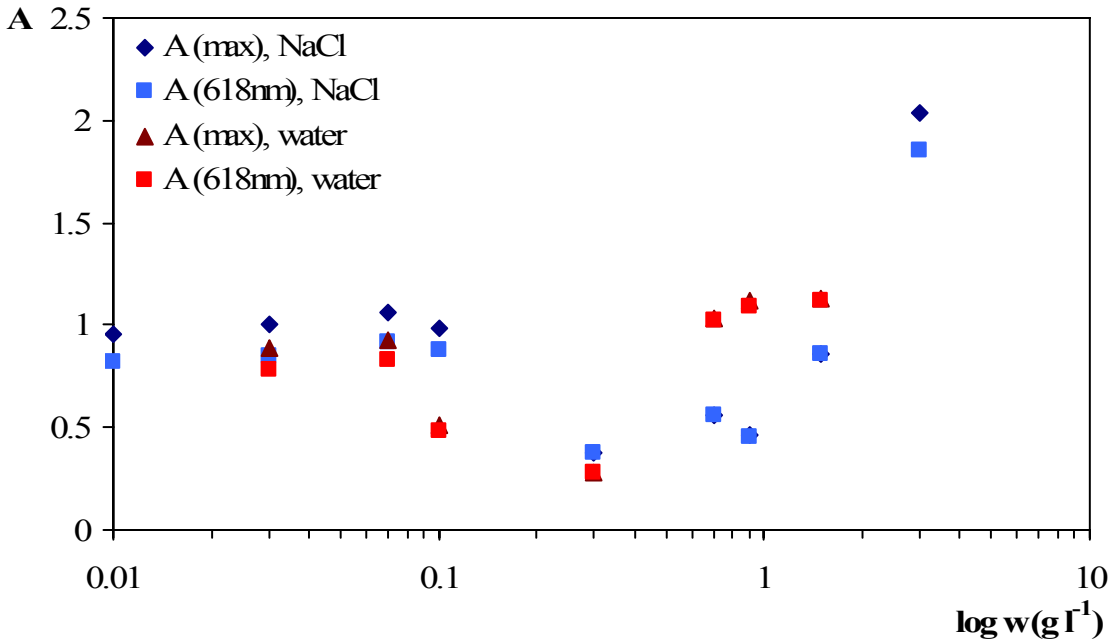

Fig. 27 *Change of absorbance of CBB in different solvents at A_{max} and A_{618nm}.*

There is the difference in absorbance values in Fig. 27. The derivative C8NHHA9 was measured in presence of water and 0.15 M NaCl to get the information about the influence of the ionic strength on the results of spectrophotometric measurement. There is an evidence of decreased absorbance in presence of NaCl, but the shape of the curves is very similar.

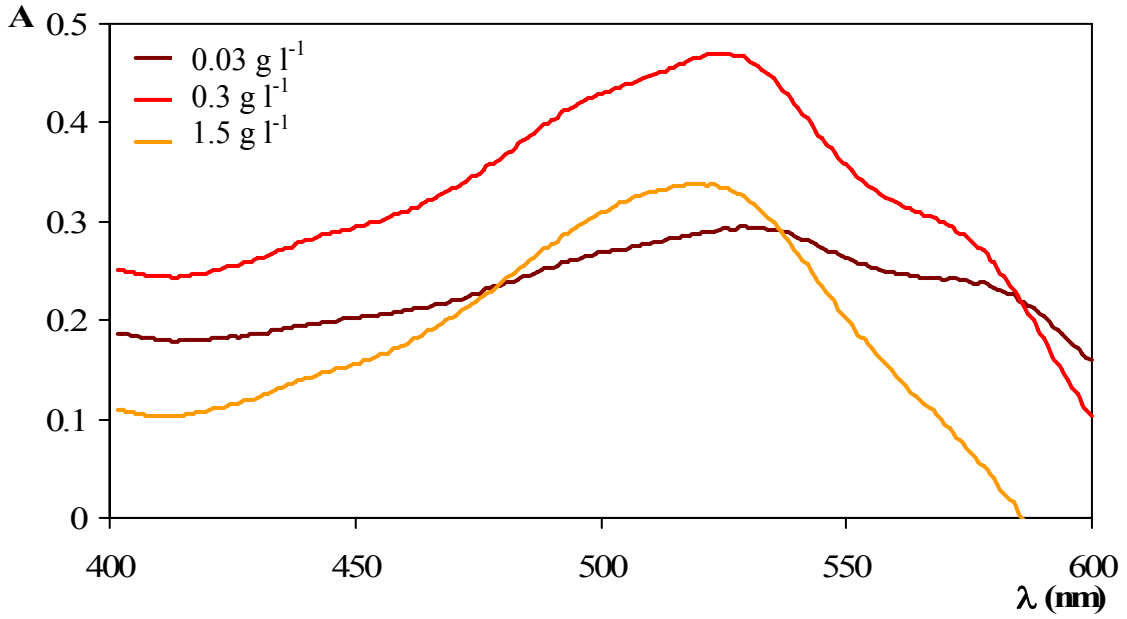

Fig. 28 *Illustration of absorption spectra of Sudan III in different concentrations of C8NHHA9 in water.*

The measurement of absorption spectra of Sudan III in presence of C8NHHA9 in water was performed at wavelength in the range of 400 to 600 nm. There is no shifting of A_{max} during the measurement observed. A_{max} increases to CAC, then there is a decreasing trend because the environment becomes apolar, which allows the higher dissolving of the water insoluble dye.

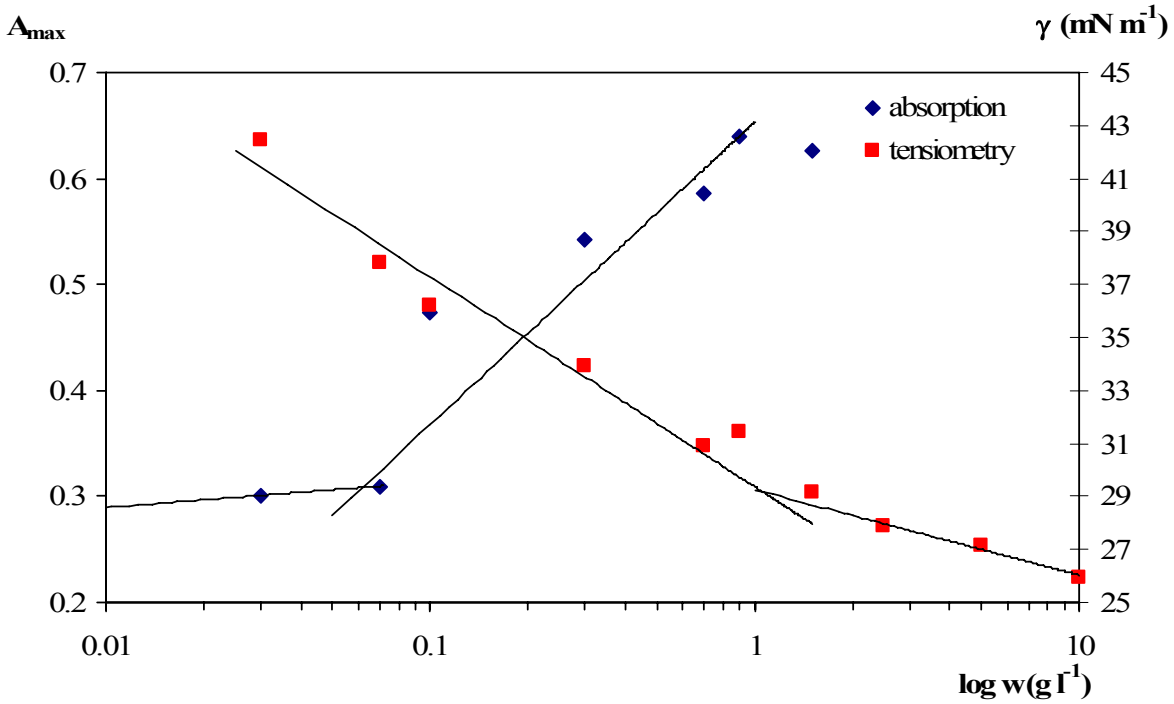

Fig. 29 *Absorption change of Sudan III at A_{max} versus logarithm of derivative concentration and surface tension of derivative solutions as a function of polymer concentration.*

The absorption change at A_{max} as a function of derivative concentration is represented in Fig. 29. The absorbance increases significantly beyond CAC. It is 0.0619 g l^{-1} obtained from the absorption measurement and 1.8 g l^{-1} obtained from tensiometry.

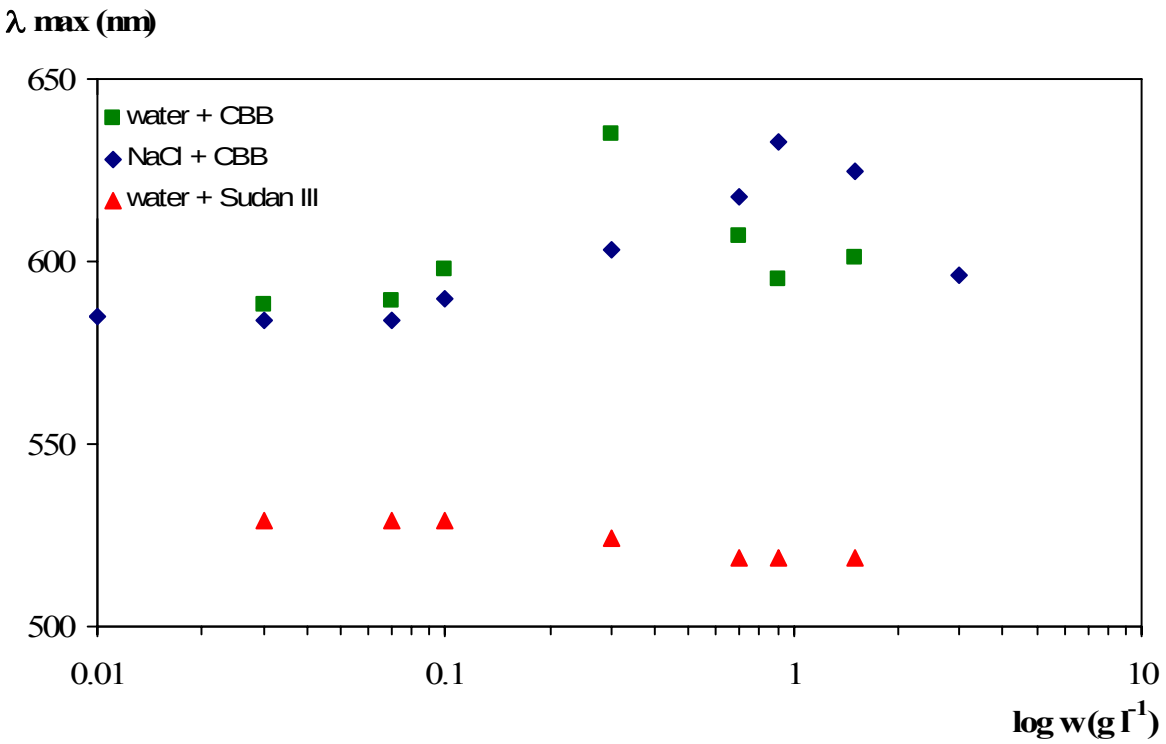

Fig. 30 *Shifting of wavelength of maximum absorption in the samples with CBB and Sudan III with derivative in NaCl and water.*

As represented in Fig. 24, A_{max} is bathochromic shifted in solutions with CBB. In adition, Fig. 30 shows change of λ_{max} vs. derivative concentration beyond CAC. It corresponds to information described in [19].

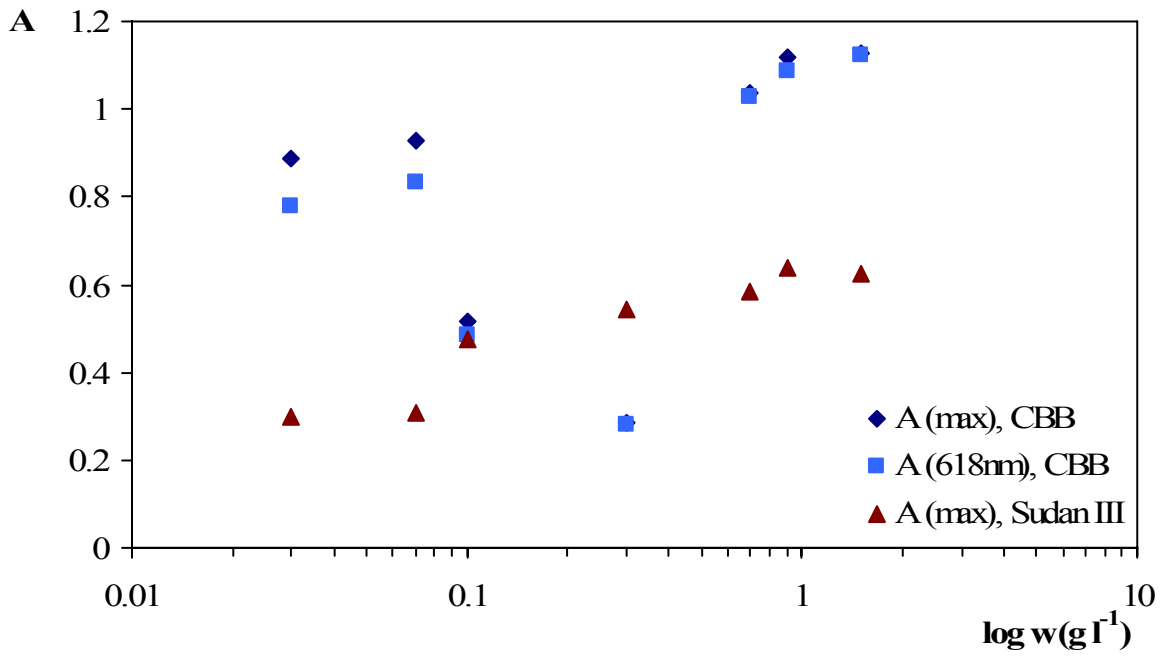

Fig. 31 *Absorbance of CBB and Sudan III.*

There is the plot of absorbance of both dyes in water, Sudan III and CBB. The dependency shows that Sudan III has at least two times lower absorbance when compared with the same samples with CBB.

Tab. 8 *Results from the solubilization measurement – CBB.*

Results for samples with CBB									
C8NHHA9 in water					C8NHHA9 in 0.15 M NaCl				
CAC (g l^{-1})	**w (g l^{-1})**	**A_{max}**	**A_{618nm}**	**λ_{max} (nm)**	**CAC (g l^{-1})**	**w (g l^{-1})**	**A_{max}**	**A_{618nm}**	**λ_{max} (nm)**
0.2897	0.03	0.3011	0.7791	588	0.6856	0.01	0.9586	0.8222	585
	0.07	0.3098	0.8322	589		0.03	1.0046	0.8457	584
	0.1	0.4734	0.485	598		0.07	1.0581	0.9181	584
	0.3	0.5418	0.2819	635		0.1	0.9811	0.8826	590
	0.7	0.5854	1.0274	607		0.3	0.3808	0.3770	603
	0.9	0.6404	1.0864	595		0.7	0.5574	0.5582	618
	1.5	0.6259	1.1224	601		0.9	0.4610	0.4563	633
						1.5	0.8554	0.8547	625
						3	2.0381	1.8546	617

All the results obtained from the solubilization measurement of CBB in hydropfobically modified hyaluronan in water and NaCl are summarized in Tab. 8. CAC were calculated from the regression method like shown in Fig. 29. The resultant value of CAC of samples in water was 0.2897 g l^{-1} and in NaCl 0.6856 g l^{-1}.

Tab. 9 *Results from the solubilization measurement – Sudan III..*

Results for samples with Sudan III			
C8NHHA9 in water			
CAC (g l^{-1})	**w (g l^{-1})**	**A_{max}**	**λ_{max} (nm)**
0.0619	0.03	0.3011	529
	0.07	0.3098	529
	0.1	0.4734	529
	0.3	0.5418	524
	0.7	0.5854	519
	0.9	0.6404	519
	1.5	0.6259	519

The increasing values of A_{max} were detected because the hydrophobic clusters are being formed it the bulk solution, which caused better solubilization of the water insoluble dye. CAC for derivative in Sudan III was determined to 0.0619 g l^{-1}. The wavelength of maximum absorbance is changed only a little, but it does not have any effect on the results.

5 CONCLUSION

One of the colliodal properties, surface tension, of hyaluronan and its hydrophobically modified derivatives was measured by Du Noüy ring method. For this purpose, surface properties of two HA varying in MW and two derivatives varying in DS and solvent were studied. As the solvent, water and chloride salts with different cations were used: NaCl, KCl, $MgCl_2$, $CaCl_2$ and $ZnCl_2$. The results shown that HA does not behave as a surface active substance, because surface tension of HA did not decreased in the range of concentration from 0.1 to 2 g l^{-1}.

On the other hand, all the samples of derivatives shown a strong reduction of surface tension due to occurence of alkyl chains bonded to the main polymer chain. Alkyl chains, which are of a hydrophobic character, are adsorbed at the surface of a water solutions and influence the values of surface tension. It is in agreement with a fact, that hydrophobic domains occur in the bulk solution and thus, CAC could be determined. The important role plays also DS, which characterizes the area occupied by a molecule of the derivate at the surface. The adsorbed polymers adopt a shrunken conformation, which is more or less condensed depending on DS. This phenomena leads to the enlargement of the interfacial molecular area.

The second part of the thesis was based on solubilization measurement of two dyes, CBB and Sudan III. It was proved by UV–VIS spectrophotometry that in both dyes environment the hydrophobically domains occur. In the case of CBB the shift of the maximum absorption peak was observed and consequently, in the case of Sudan III, the increase of absorbance was detected. It corresponds to better solubilization of water insoluble dye Sudan III.

All the results except HA in itself are in connection with the idea to use such a modified hyaluronan as a drug delivery system for drugs with the hydrophobic character, because the clusters formed in the bulk solution should allow to deliver these drugs directly to the place in an organism.

6 LIST OF ABBREVIATIONS

HA	Hyaluronic acid
MW	Molecular weight
Da	Dalton, unit of MW
CD44	Type of HA receptor
RHAMM	Type of HA receptor
LAYLIN	Type of HA receptor
HARE	Type of HA receptor
LYVE-1	Type of HA receptor
CDC37	Type of HA receptor
HMW HA	High molecular weight HA
LMW HA	Low molecular weight HA
HM	Hydrophobically modified
DS	Degree of substitution
DMF	Dimethylformamide
CMC, CAC	Critical micelle concentration, critical aggregation concentration
SDS	Sodium dodecyl sulphate
N	Aggregation number
$\tau_{1,2}$	Relaxation times in micellization process
CBB	Coomassie Brilliant Blue
γ	Surface tension
F	Force exerted parallel to the surface
L	Length of the surface
a	Constant derived from the capillary pressure
r_p	Radius of the Du Noüy ring
Φ	Correction factor for surface tension calculation
F_{max}	Maximum force at Wilhelmy plate method
F_V	Weight of lifted volume of liquid
L_W	Wetted length
θ	Contact angle
M, c	Molar concentration, mol l^{-1}
C8NHHA9	Hydrophobically modified derivative of hyaluronan
C8NHHA10	Hydrophobically modified derivative of hyaluronan
t	Time of measurement
w	Mass concentration, g l^{-1}
γ_0	Surface tension of a solvent
Γ	Maximum surface concentration
A_m	Surface area occupied by one molecule
pC_{20}	Efficiency
Π_{CAC}	Effectiveness at CAC
R	Gas constant
T	Kelvin Temperature
k	slope of a regression equation
N_A	Avogadro's number
K	Intercept of a regression equation
γcac	Surface tension at CAC

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